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APPLICATION NUMBER: *60/399,708*
FILING DATE: *August 01, 2002*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

Docket Number	02/24051	Type a plus sign (+) inside this box →	+

INVENTOR(s) / APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
TEICHBERG	Vivian	I.	Savyon, Israel

TITLE OF THE INVENTION (280 characters max)

METHOD OF REDUCING BRAIN GLUTAMATE LEVELS, IN PARTICULAR AS A TREATMENT OF NEURODEGENERATIVE DISEASES

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification	*Number of Pages*	61	☑ Applicant is entitled to Small Entity Status	
☑ Drawing(s)	*Number of Sheets*	26	☐ Other (specify)	

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☐ A check or money order is enclosed to cover the filing fees			
☑ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	50-1407	FILING FEE AMOUNT ($)	$ 80.-

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

☐ Yes, the name of the US Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date August 1, 2002

TYPED or PRINTED NAME SOL SHEINBEIN

25,457
REGISTRATION NO. *(if appropriate)*

☐ Additional inventors are being named on separately numbered sheets attached hereto

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

METHOD OF REDUCING BRAIN GLUTAMATE LEVELS, IN PARTICULAR AS A TREATMENT OF NEURODEGENERATIVE DISEASES

Inventor: Vivian TEICHBERG

Content:

A. IN VITRO SCAVENGING OF BLOOD GLUTAMATE BY ACTIVATION OF RESIDENT ENZYMES

Abstract

Several intractable brain pathological conditions are characterized by the presence of excess Glutamate in brain interstitial fluid. In order to increase the driving force for an enhanced brain-to-blood efflux of Glutamate, we have investigated here the in vitro conditions allowing a decrease of blood Glutamate levels. Accordingly, we have examined the results of the activation of the blood resident enzymes, glutamate-pyruvate transaminase (GPT), glutamate-oxaloacetate transaminase (GOT) and glutamate dehydrogenase (GDH). The activation of GOT and GPT by repetitive additions to blood of oxaloacetate and pyruvate respectively causes a decrease of Glu levels up to 60% mainly in the blood cell compartment but also in plasma. These effects are potentiated by thiaminepyrophosphate and lipoamide, two cofactors of the 2-ketoglutarate dehydrogenase. The activation of GDH by NAD leads to a decrease of Glu levels only in the blood cell compartment.

Introduction

The amino acid L-glutamic acid (Glu), mediates most of the excitatory transactions between neurons in the central nervous system. While its normal functioning allows one to normally move, feel, perceive, learn and memorize, its perturbed function may lead to cognitive, affective, sensory or motor deficiencies that result from Glu neurotoxicity (Choi, 1992). The potent neurotoxic (also called excitotoxic) properties of Glu find their expression in the various pathological conditions in which an excessive release of L-Glu takes place. These include acute brain anoxia/ischemia i.e stroke (Graham et al., 1993; Castillo et al., 1996), perinatal brain damage (Hagberg et al., 1993; Johnston, 1997), traumatic brain injury (Baker et al., 1993; Zauner et al., 1996), bacterial meningitis (Spranger et al, 1996), subarachnoid hemorrhage (Sarrafzadeh et al. 2002), open heart and aneurysm

surgery (Persson et al., 1996; Saveland et al., 1996), hypothermic circulatory arrest (Tseng et al. 1999) newly diagnosed epilepsy (Kalviainen et al., 1993), glaucoma (Dreyer et al., 1996), acute liver failure (Rose et al. 2000) and various chronic neurodegenerative diseases such as amyotrophic lateral sclerosis (Rothstein et al., 1990; Shaw et al., 1995), HIV dementia (Ferrarese et al. 2001) and Alzheimer's disease (Pomara et al., 1992).

It is today widely believed that manipulations of the glutamate system offer potentially beneficial opportunities for a management of the acute or chronic neurological deficits mentioned above. On these premises, Glu receptor antagonists have been developed and found to display potent neuroprotective properties in animal models but have not been used so far in the clinic because of their unacceptable side effects. Attempts have also been made to increase the activity of the various glutamate transporters, present on glia and neurons, which take up Glu from the extraneuronal fluid and limit thereby Glu excitatory action and excitotoxicity. As none of the above approaches have been successful so far, alternative strategies ought to be evaluated. Along these lines, we have made the hypothesis that it might be feasible to boost a brain-to-blood efflux of Glu, as was attained for example in the case of asparagine (Gentili et al. 1996), by a decrease of its blood levels. The administration to blood of asparaginase decreased the levels of asparagine both in plasma and in the cerebrospinal fluid (CSF).

We have investigated here the possibility of achieving similarly a decrease of blood Glu levels by the activation of the blood resident enzymes, glutamate-pyruvate transaminase (GPT), glutamate-oxaloacetate transaminase (GOT) and glutamate dehydrogenase (GDH). These enzymes convert L-glutamate into 2-ketoglutarate. With GPT and GOT, the respective glutamate co-substrates pyruvate (Pyr) and oxaloacetate are transaminated into alanine and aspartate. We present here results defining the in vitro conditions for reducing Glu in blood

Materials and Methods

Glutamate dehydrogenase was from Roche; glutamate-pyruvate transaminase was from Sigma. All chemicals were purchased from Sigma unless noted otherwise

Blood was collected retroorbitally from 200-250 g Sprague Dawley rats anaesthesized intraperitoneally with 40 mg ketamine and 5 mg xylazine/kg body weight. Fresh rat blood was incubated at 37°C in the presence of pyruvate, oxalacetate adjusted to pH 7.4, or a combination of both. Every 15 min, when needed, blood was supplemented with these substrates (freshly prepared 100 mM stock solutions) to obtain a final concentration of 1mM.

Aliquots of 1 microliter were removed at each time point and centrifuged at 1,300×g for 7 min. The volume of supernatant (plasma) was measured and an identical volume of 1M PCA (perchloric acid) was added to precipitate proteins. The mixture was left for 10 min on ice and centrifuged. The cell pellet (erythrocytes, lymphocytes, platelets) was lysed by osmotic shock upon resuspension in double distilled water up to a final volume of 1microliter and an identical volume of 1M PCA was added. Both plasma and cell PCA-precipitated fractions were centrifuged at 16,000×g for 10 min and the pellet discarded.

Glutamate concentration was measured in the supernatant using the fluorometric method of Graham and Aprison (1966). A 20microliter aliquot from PCA supernatant was added to 480microliter HG buffer containing 15 U of glutamate dehydrogenase in 0.2mM NAD, 0.3 M glycine, 2.4% hydrazine hydrate adjusted to pH 8.6 with 1N H_2SO_4 . After incubation for 30-45 min at room temperature, the fluorescence was measured at 460 nm after excitation at 350 nm. A glutamate standard curve was established with concentrations ranging from 0-6 micromolar. All determinations were done in duplicates.

Results

Since GPT and GOT catalyze equally well both the degradation of Glu (into 2-ketoglutarate- forward reaction) and its synthesis (from 2-ketoglutarate –back reaction), we investigated whether the administration of Pyr and oxaloacetate to blood results in a displacement of the enzymatic equilibria towards the degradation of Glu.

Figure 1A illustrates the evolution of the blood levels of Glu under various incubation conditions at 37°C. The filled diamonds shows that the Glu levels in blood increase monotously when the latter is simply incubated at 37°C. This trend increases very significantly when blood is supplemented at t=0 with 2.5 u/ml GPT (empty circles) indicating that significant pools of 2-ketoglutarate and alanine are normally present in blood to drive the synthesis of Glu. The trend however completely reverses when Pyr, at a final concentration of 1 mM, is added at t=0 in the absence (closed squares and black arrow) or presence of 2.5u/ml GPT (closed circles) or at t= 0, 15, 30 min (empty squares and black arrows). These results indicate that the activation by Pyr of the blood endogenous GPT causes a decrease of Glu levels. This process however is transient because soon after the consumption of Pyr (about 15-20 min after its last addition to blood) and the concomitant production of alanine and 2-ketoglutarate, the latter apparently is converted back into Glu.

Figure 1B illustrates the evolution of the blood levels of Glu upon supplementation at t= 0, 15, 30 min (arrows) of 1 mM Pyr (closed squares), 1 mM oxaloacetate (closed triangles) or a mixture of 1mM Pyr and 1 mM oxaloacetate (closed diamonds). The activation of GOT by oxaloacetate causes a faster and larger decrease of blood glutamate than that caused by Pyr, and no synergism is observed. This suggests that the GOT/GPT-mediated conversion of Glu has reached a maximal extent that is limited by the concomitant 2-ketoglutarate concentration build-up that drives the GPT and GOT back reactions.

As blood contains two major pools, a cell pool consisting of various cells mainly erythrocytes, leukocytes and platelets and a plasma pool, it was of interest to

determine the fate of Glu in these two pools following the activation of endogenous GPT and GOT. Figure 2A shows the evolution of the Glu concentration in the cell pool (closed symbols) and in plasma (open symbols) following the repeated additions (at arrows) to blood of 1mM Pyr (squares), 1 mM oxaloacetate (triangles) or of a mixture of 1mM Pyr and 1 mM oxaloacetate (diamonds). The additions of Pyr or oxaloacetate cause a similar reduction in the cell Glu pool but while Pyr increases the Glu plasma pool, oxaloacetate decreases it. When combined, Pyr and oxaloacetate have a synergistic action and decrease both pools of Glu. Figure 2B shows that a similar effect is observed when Pyr and oxaloacetate are added following the separation of the pools. In this case, the cell pool was separated from plasma by centrifugation, washed and resuspended in PBS. The results obtained suggest that, once taken up into the cell pool presumably by monocarboxylate transporters, Pyr and oxaloacetate are able to activate the intracellular GPT and GOT. As the Glu concentration in the cell pool (120+/- 4.9 micromolar) is about 3 times that of plasma (40.8+/- 5.8 micromolar), the decrease of the cell pool is expected to account principally for the decrease of blood Glu observed in figure 1. Analysis of the initial rates of Glu conversion shows that the rates in the cell pool (3.3%/min) is about 2.5 times that in plasma (1.3%/min). These rates are compatible with the Glu concentrations in the respective pools and in line with the Glu Km values of GOT/GPT in the mM range assuming that the levels of these enzymes are similar in both pools. Because of the synergistic actions of Pyr and oxaloacetate in decreasing Glu, all the studies hereafter were performed using mixtures of the co-substrates of GPT and GOT.

Figure 3 shows the extent of Glu conversion reached after 60 min following the addition to blood of increasing concentrations of a mixture of Pyr and oxaloacetate. It can be seen that half of the maximal effect is observed at a submillimolar concentration in line with the known Km values for Pyr and oxaloacetate, and saturation of the effect takes place at about 5 mM

As both GPT and GOT utilize pyridoxal phosphate as cofactor, we also tested whether the addition of 15 micromolar pyridoxal phosphate enhances the ability of Pyr and oxaloacetate to decrease the cellular or plasma Glu concentration but no significant effects of pyridoxal phosphate were observed (data not shown).

In view of the fact that Glu is converted into 2-ketoglutarate and that the latter can drive the GOT and GPT back reactions and limit thereby the conversion of Glu, we investigated whether 2-ketoglutarate, the common co-product of GOT and GPT, could possibly serve as a substrate of the 2-ketoglutarate dehydrogenase which converts it into succinyl CoA in the presence of CoA and NAD. Since thiamine pyrophosphate and lipoic acid are cofactors of the 2-ketoglutarate dehydrogenase, we tested whether their addition to blood increases the extent of Glu conversion resulting from the combined addition of Pyr and oxaloacetate.

Figure 4 shows the extent of Glu conversion reached after 60 min following the repeated additions to blood of increasing concentrations of lipoamide (Fig4 upper graph) and thiaminepyrophosphate (Fig4 lower graph) in the presence of a mixture of 1mM Pyr and 1mM oxaloacetate. It can be seen that both cofactors of the 2-ketoglutarate dehydrogenase increase the conversion of Glu by about 15-20%.

The results presented in Figure 5 establish that both thiaminepyrophosphate (upper graph) and lipoamide (lower graph) increase the initial rates of Glu conversion (in a range of 20-50%) as well as its extent, in line with the concept that the activation of the 2-ketoglutarate dehydrogenase facilitates the GOT/GPT-mediated Glu scavenging.

We also investigated the possible contribution of glutamate dehydrogenase GDH to glutamate conversion. GDH is a multimeric enzyme that uses NAD or NADP as cofactors to transform Glu into 2-ketoglutarate and ammonia. It is allosterically activated by ADP and leucine. We therefore tested the effects of the addition to blood of NAD, ADP and leucine (all at 1mM) either separately or in combination. Figure 6 illustrates the effects of the repeated addition to blood of NAD and the subsequent analysis of Glu levels in the cell and plasma compartments: while

a decrease of Glu levels was found to take place in the blood cellular compartment, an increase of Glu took place in plasma. In all instances when blood was incubated with NAD, Leu or ADP either separately or in combinations, increases in plasma Glu levels took place. However, systematic decreases in the cellular compartment was observed when NAD was present alone or in combination with Leu and ADP while the latter on their own did not significantly affect Glu levels. These results could be interpreted by suggesting that NAD may possibly activate GDH in the cellular compartment and the 2-ketoglutarate produced would be released from the cells and converted back into Glu via the GOT or GPT present in the plasma. It is clear however that the activation of GDH is not a suitable mean to decrease plasma Glu.

Discussion

The possibility of reducing the brain levels of Glu by reducing its concentration in blood is an approach that ought to be investigated in view of the present failures to develop a successful drug-mediated control of the excitotoxic damage caused by excess Glu in both acute and chronic neurodegenerative conditions. Several examples are available to show that the decrease of amino acids in blood reduces in parallel their concentration in the cerebrospinal fluid. The administration of asparginase in the treatment of children with acute lymphoblastic leukemia (Avramis et al. 2002) or of sodium benzoate (Wolff et al. 1986) in the management of seizures in nonketotic hyperglycinemia, reduce the respective concentrations of asparagine and glycine both in plasma and CSF (Woo et al. 2000;.Wolff et al. 1986).

To reach a similar objective but for Glu, we made here the first necessary step which is to define the in vitro conditions under which Glu concentration can be reduced in blood and in plasma. It is expected, but not yet proven, that a decrease in the plasma Glu concentration provides the driving force for an accelerated brain to blood Glu efflux.

We observed that the basal levels of the blood resident transaminases GPT and GOT are large enough in order to convert Glu into 2-ketoglutarate in the presence of Pyr and oxaloacetate respectively. The latter compounds are clearly utilized as substrates since repetitive additions to blood are needed to produce a decline of Glu levels. This effect is not sustained because of the reversibility of GOT and GPT reactions. One may argue that this reversibility is not a drawback in a therapeutic context where a fast and neuroprotective decrease of Glu levels in brain is needed, since such an effect might be achieved for the entire duration of Pyr and oxaloacetate administrations if indeed the decrease of plasma Glu concentration causes an accelerated brain to blood Glu efflux.

Pyr and oxaloacetate exert a synergistic action in which oxaloacetate and GOT have a dominant contribution. Pyr decreases the concentration of blood Glu because the latter consists of a dominant cellular pool, which is reduced by Pyr, and of a minor plasma pool that is surprisingly increased by Pyr. We have nor investigated the mechanism responsible for this increase but one can surmise that it originates from a GPT induced formation of 2-ketoglutarate which, in the presence of plasma aspartate, can drive the synthesis of Glu via GOT activation. To minimize this contribution of GOT, it is necessary to activate both GPT and GOT in the direction of the formation of 2-ketoglutarate. By combining Pyr and oxaloacetate to thiamine pyrophosphate and/or lipoamide, the coenzymes of the 2-ketoglutarate dehydrogenase, one further facilitates the irreversible conversion of Glu since its precursor 2-ketoglutarate is eliminated by conversion into succinylCoA and CO_2.

The fact that the addition of Pyr and oxaloacetate to blood decreases Glu both in the cellular pool and in the plasma pool may be surprising at first. However, GOT and GPT are present in both pools, and erythrocytes harbor monocarboxylate transporters MCT1 (Garcia et al. 1994) that readily allow an influx of Pyr and oxaloacetate. For a potential therapeutic effect, the decrease of the Glu plasma concentration is the important objective to achieve because it is the latter that sets up the extent of the brain to blood Glu concentration gradient across the brain capillary

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endothelial cells. The present study suggests that the addition to blood of submillimolar concentrations of Pyr and oxaloacetate in combination with either lipoamide or thiaminepyrophosphate is a suitable procedure to decrease plasma Glu. It remains to be established whether this procedure is equally suitable under in vivo conditions.

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Lends of figures

Figure 1

A. Evolution of Glutamate blood levels under various incubation conditions at 37°C. Control blood (filled diamonds). Addition of 2.5 u/ml GPT (empty circles). Addition of 1mM Pyruvate at t=0 in the absence (closed squares and black arrow) or presence of 2.5u/ml GPT (closed circles) or at t= 0, 15, 30 min (empty squares and black arrows). Each point represents the average of at least two Glu determinations.

B. Evolution of Glutamate blood levels upon supplementation at t= 0, 15, 30 min (arrows) of 1 mM Pyr (closed squares), 1 mM oxaloacetate (closed triangles) or a mixture of 1mM Pyr and 1 mM oxaloacetate (closed diamonds). Glu concentration at t=0 was 166± 13 micromolarol/l. Each point represents the average of at least two Glu determinations.

Figure 2

A. Evolution of the Glu concentration in blood cellular pool (closed symbols) and in plasma (open symbols) following the repeated additions (at arrows) to blood of 1mM Pyr (squares), 1 mM oxaloacetate (triangles) or of a mixture of 1mM Pyr and 1 mM oxaloacetate (diamonds). Each point represents the average of at least two Glu determinations.

B. Effects of Pyr and oxaloacetate as in A following separation of blood into cellular and plasma pools. Each point represents the average of at least two Glu determinations.

Figure 3

Extent of Glu conversion reached after 60 min following the addition to blood of increasing concentrations of an equimolar mixture of Pyruvate and oxaloacetate. Analysis of the cellular pool (filled diamonds) and plasma (open diamonds). Each point represents the average of at least two Glu determinations.

Figure 4

Extent of Glu conversion reached after 60 min following the repeated additions to blood of increasing concentrations of lipoamide (upper graph) and thiaminepyrophosphate (lower graph) in the presence of a mixture of 1mM Pyr and 1mM oxaloacetate. Analysis of the cellular pool (filled diamonds) and plasma (open diamonds). Each point represents the average of at least two Glu determinations.

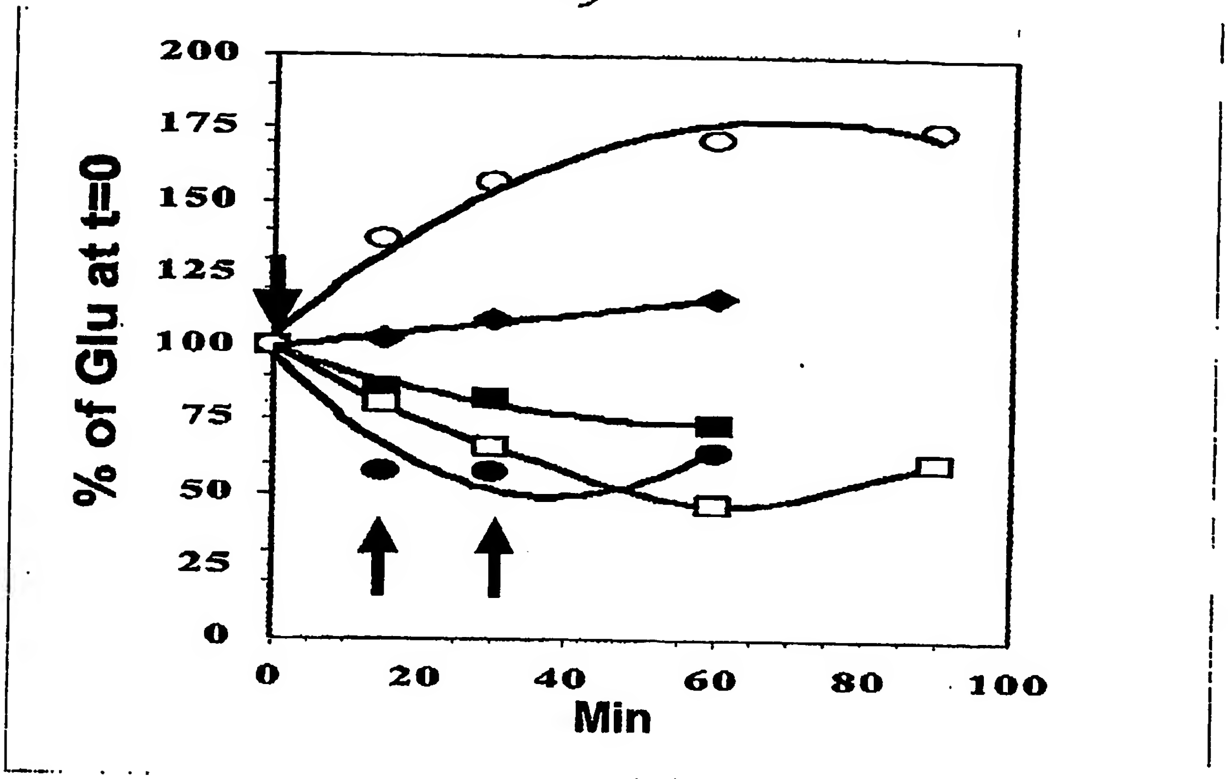
Figure 5

Effects of 10 micromolar thiaminepyrophosphate (upper graph) and 0.5 mM lipoamide (lower graph) on the evolution of Glu conversion produced by a mixture of 1mM Pyr and 1mM oxaloacetate. Analysis of the cellular pool (filled diamonds- control; filled circles- addition of 10 micromolar thiaminepyrophosphate (upper graph) or 0.5 mM lipoamide (lower graph)) and plasma (open diamonds- control; open circles- addition of 10 micromolar thiaminepyrophosphate (upper graph) or 0.5 mM lipoamide (lower graph)). Each point represents the average of at least two Glu determinations.

Figure 6

Effects of the repeated addition to blood of NAD and the subsequent analysis of Glu levels in the cell and plasma compartments. Analysis of the cellular pool (filled squares) and plasma (open squares). Each point represents the average of at least two Glu determinations.

Fig 1A



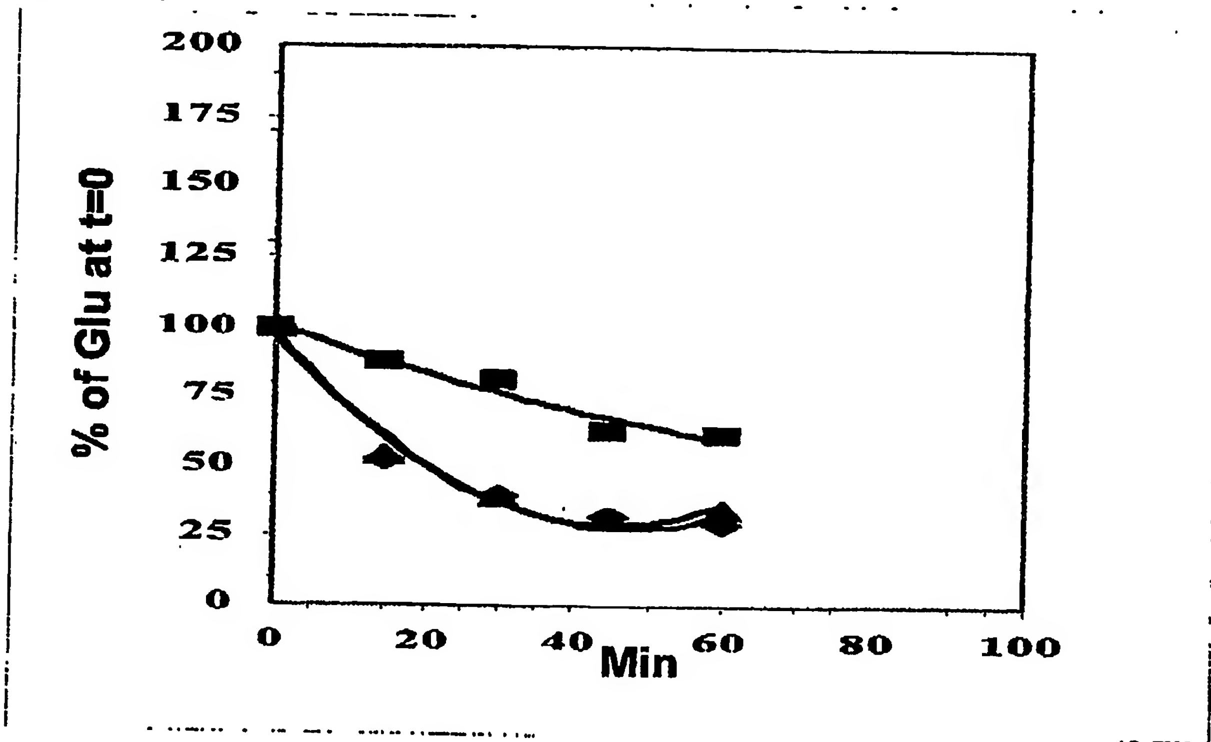


Fig. 1B

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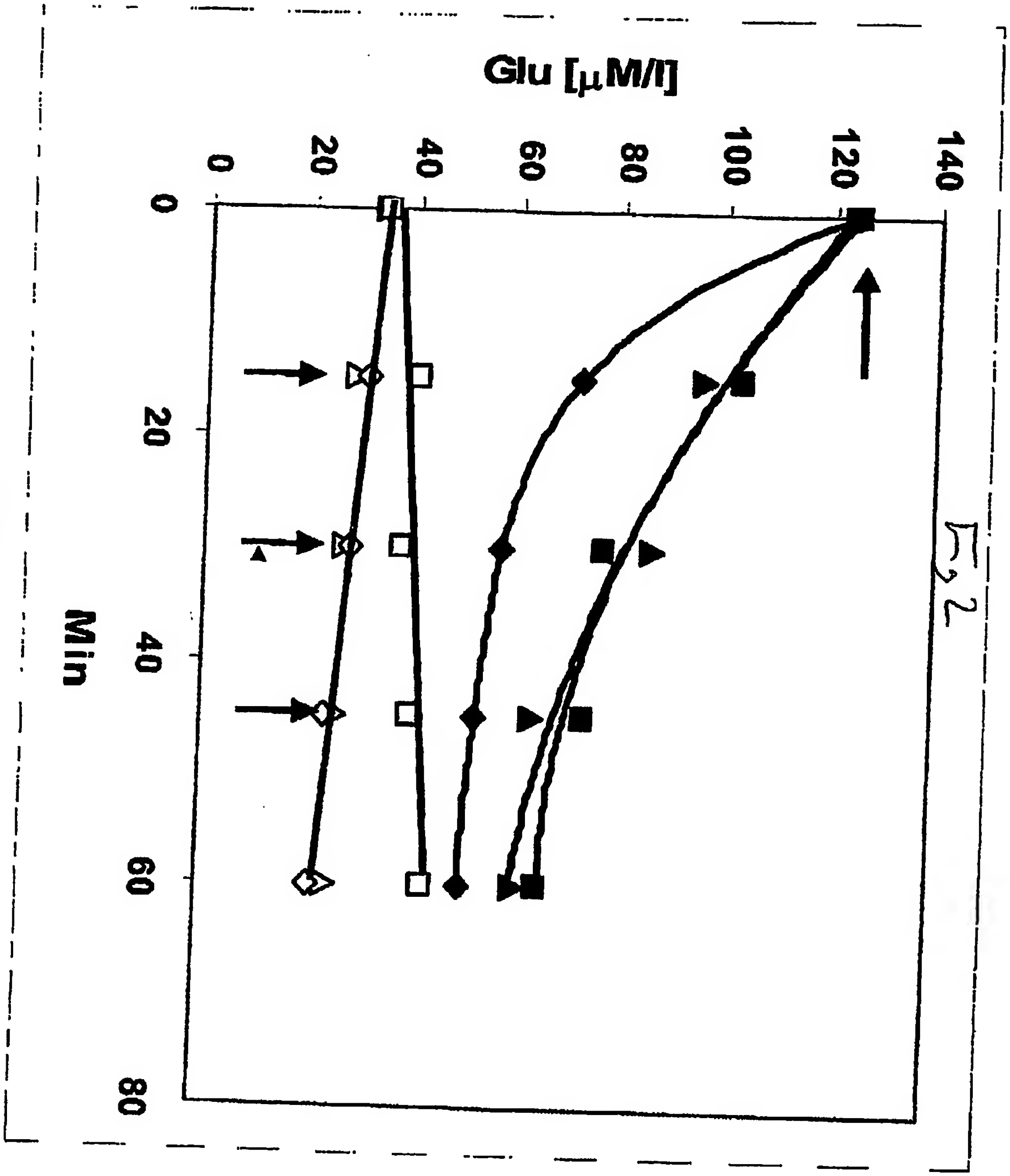

Fig 2

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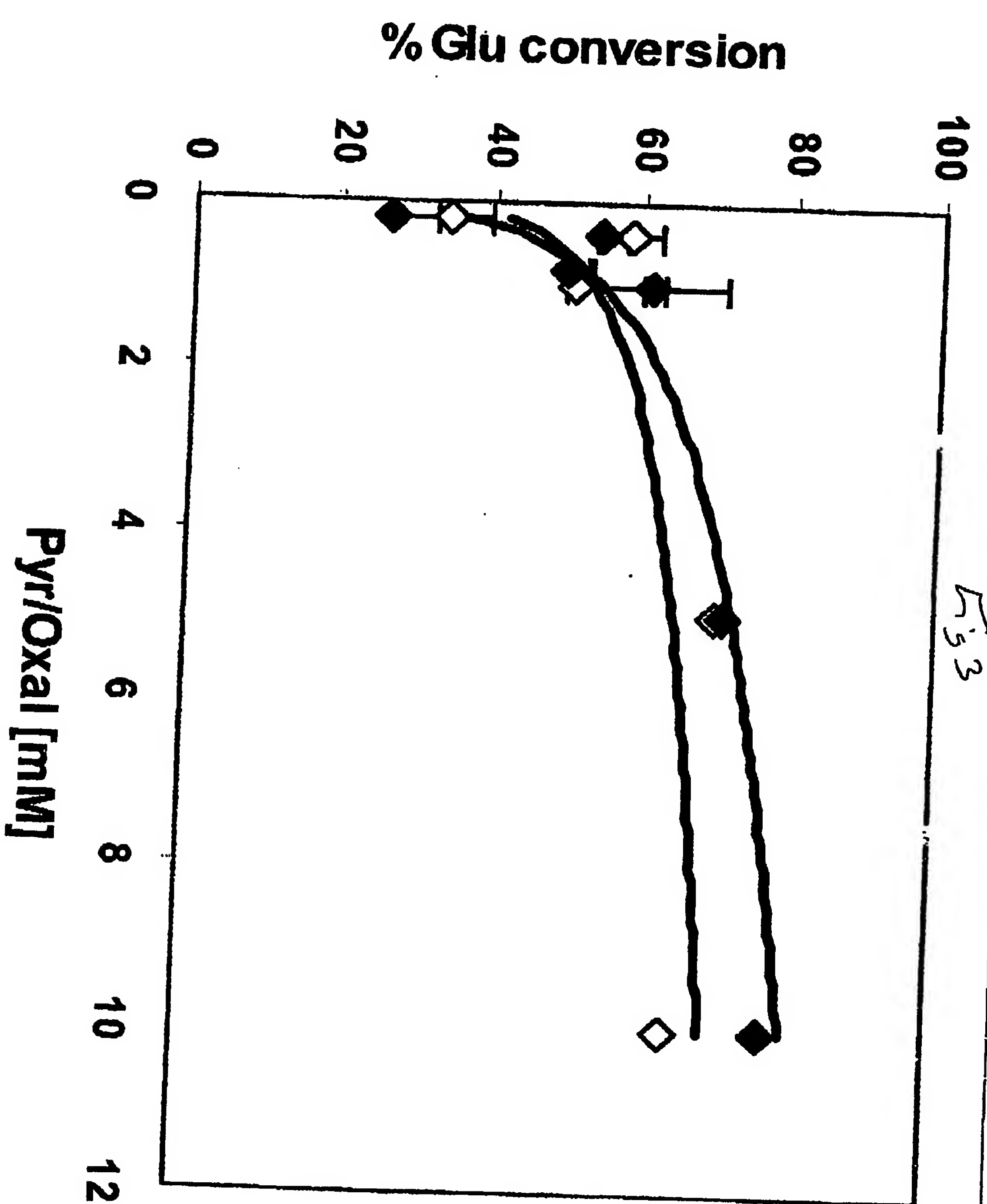


Figure 4a

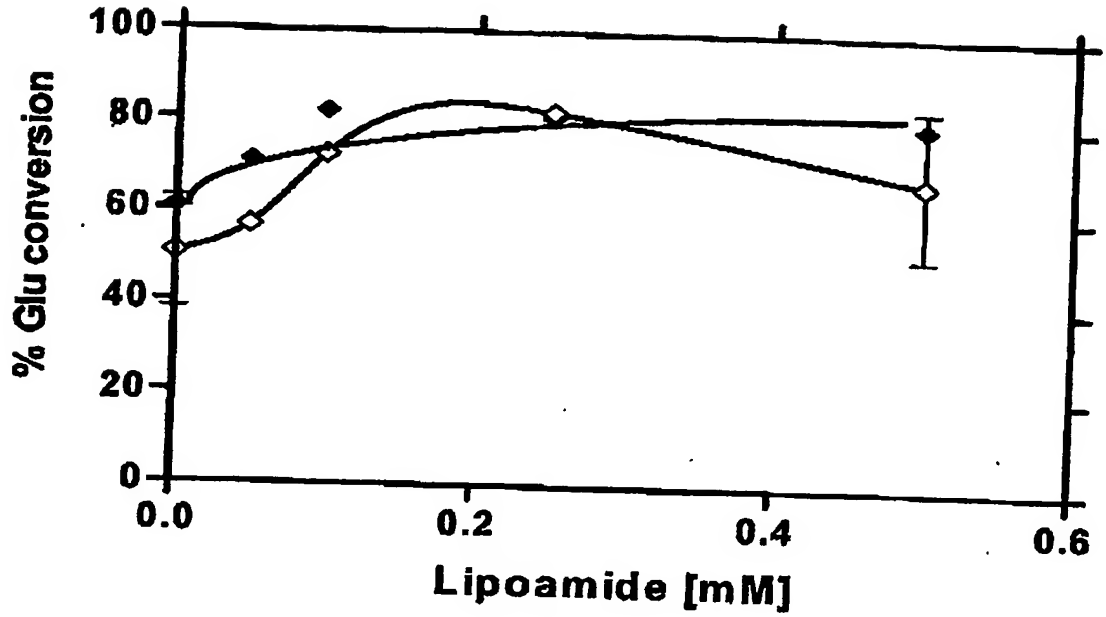


Figure 4b

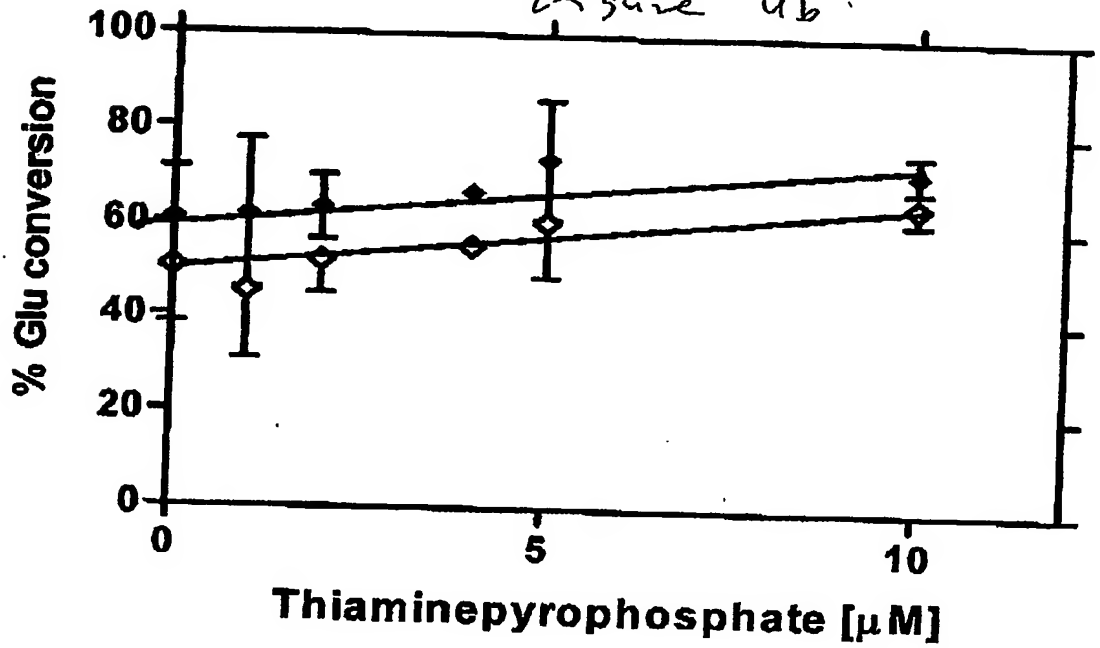
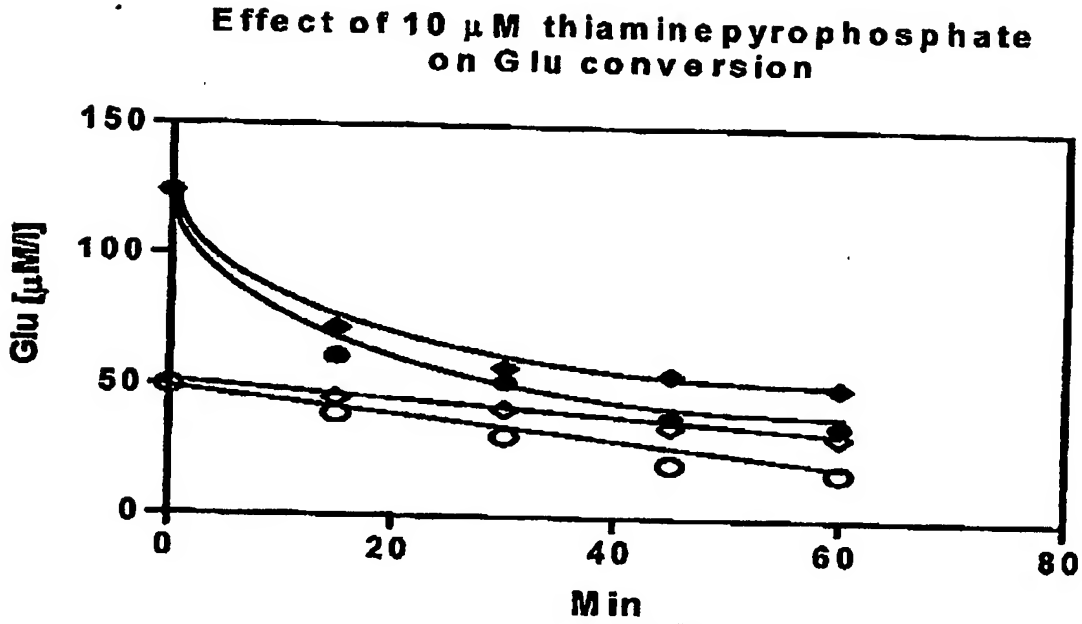


Fig. 5A


Effect of 10 µM thiaminepyrophosphate
on Glu conversion
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150
100
50
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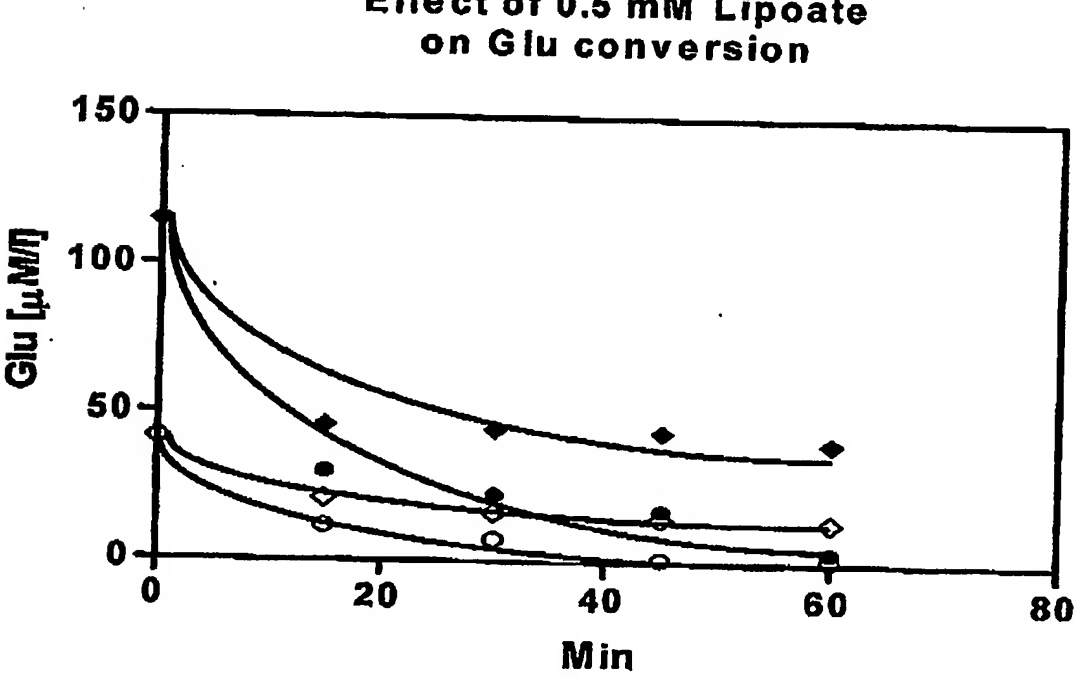
40
60
80
Min

Fig. 5B


Effect of 0.5 mM Lipoate
on Glu conversion
Glu [µM/l]
150
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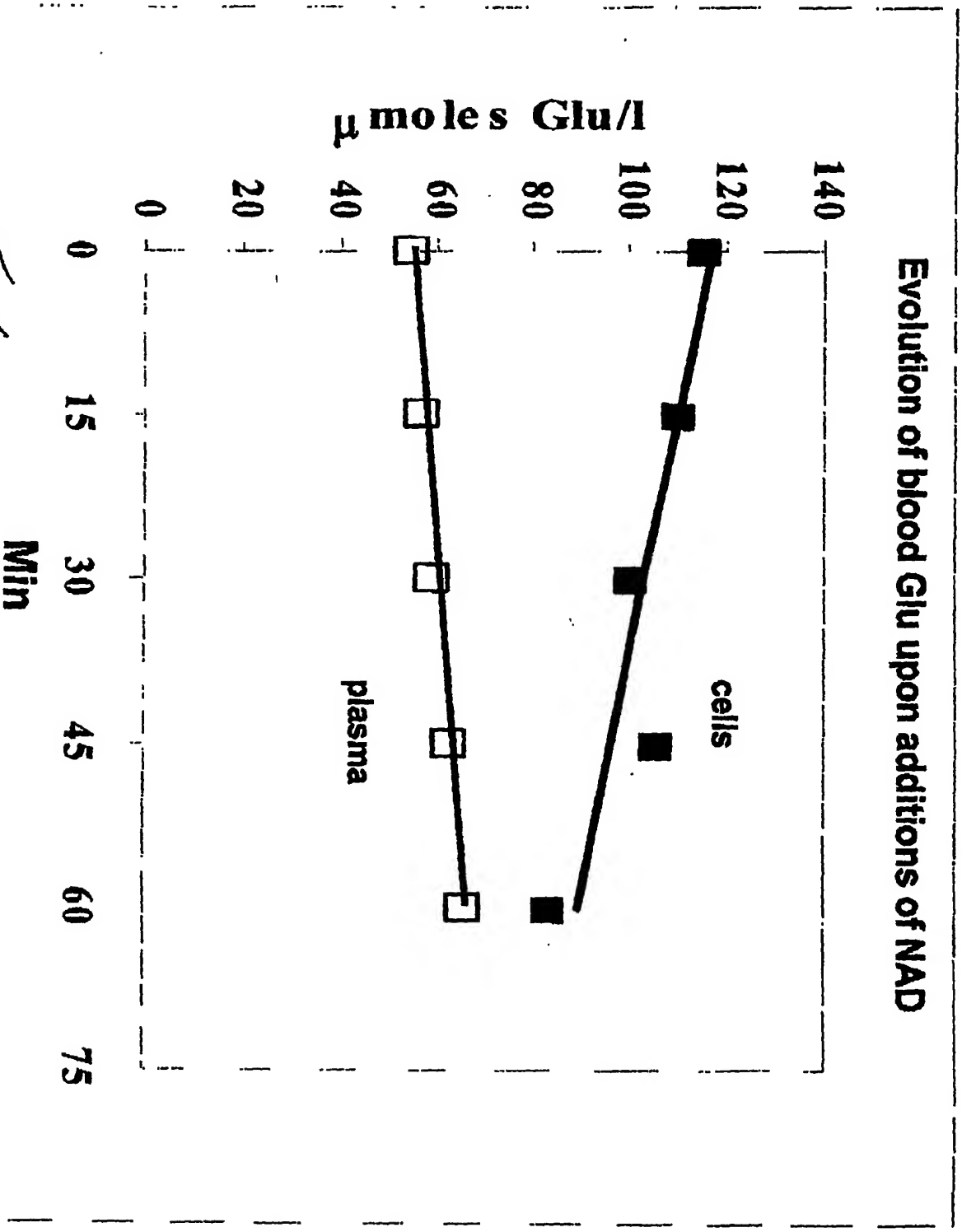
Evolution of blood Glu upon additions of NAD
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120
140
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plasma
cells

Fig-6

B. IN VIVO SCAVENGING OF BLOOD GLUTAMATE BY ACTIVATION OF RESIDENT ENZYMES

Abstract

Several intractable brain pathological conditions are characterized by the presence of excess Glutamate in brain interstitial fluid. In order to increase the driving force for an enhanced brain-to-blood efflux of Glutamate, we have investigated here the in vivo conditions allowing a decrease of blood Glutamate levels. We have found that the activation of the blood resident enzymes, glutamate-pyruvate transaminase (GPT), glutamate-oxaloacetate transaminase (GOT) by intravenous administration of their respective co-substrates pyruvate and oxaloacetate causes a decrease of blood cell and plasma Glu levels up to 50%. Upon cessation of the administration of the GOT/GPT co-substrates, their blood concentration decrease and that of Glu increases to levels significantly higher than control basal Glu levels. The latter process is discussed and attributed to a compensatory Glu efflux into blood from various organs including brain.

Introduction

Several neurological disorders such as stroke (Castillo et al. 1996), head trauma Zauner et al. 1996), amyotrophic lateral sclerosis (Shaw et al. 1995) and AIDS dementia (Ferrarese et al. 2001) are characterized by the presence of excess levels of glutamate in brain interstitial fluid. At the elevated levels it reaches, glutamate exerts excitotoxic properties (Choi, 1992) that initiate or contribute to the neurodegenerative process observed in these disorders. Since glutamate excitotoxicity is mediated by the glutamate receptors, a potential therapeutic approach has been to develop and apply various selective glutamate receptor antagonists in animal models of neurodegeneration. Though displaying powerful neuroprotective effects in experimental stroke and head trauma, the glutamate

receptor antagonists failed in clinical trials mainly because of their adverse or even lethal effects (Birmingham, 2002; Lutsep and Clark ,2001; Palmer, 2001).

These failures led us to propose an alternative approach to the neutralization of the deleterious effects of glutamate, which is to cause an increased pumping into blood of brain interstitial fluid glutamate. Our approach is based on the following facts: blood capillaries run through brain at an extremely high density and are on average no more than 10 micron away from any neuron (Pawlik et al. 1981). The endothelial cells, that form the walls of the brain blood capillaries, harbour glutamate transporters on their antiluminal side (brain side), and have the ability to take up glutamate (O'Kane et al. 1999) and concentrate it into their cytoplasm at concentrations that exceeds blood glutamate levels. Glutamate can then flow into blood down its concentration gradient via facilitated diffusion (Lee et al. 1998). According to this scheme, decreasing blood glutamate concentration should ease the brain to blood glutamate efflux.

In the first paper of this series (Yin et al. 2002), we established the in vitro conditions under which the activation of the blood resident enzymes glutamate pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) with their respective co-substrates pyruvate and oxaloacetate, causes a decrease of blood glutamate concentration.

In the present paper, we have investigated the conditions enabling a decrease of blood glutamate in vivo.

Materials and Methods

Glutamate, sodium pyruvate, sodium oxaloacetate, NADH, lactate dehydrogenase and malate dehydrogenase were from Sigma. Glutamate dehydrogenase was from Boeringer.

Sprague-Dawley rats (250-300g) were anaesthesized with an intraperitoneal injection of 40 mg ketamine and 5 mg xylazine/kg body weight. Catheterization of the tail vein (for drug injections) and of the femoral vein (for blood aliquots

withdrawals) were performed using PE10 polyethylene tubings linked to PE50 polyethylene tubing. All catheters were secured with 5-0 silk thread and flushed with heparin (3-5 microliter of 182 U/ml). Body temperature was maintained with a lamp and rectal temperature was monitored. Rat pulse rate was monitored using a Periflux system 500 and a laser Doppler probe placed onto the skull.

Intravenous injections of the various compounds diluted in phosphate buffered saline (PBS) were carried out at a rate of 0.05 ml/minute for 30 min with a Pharmacia pump P-1. During injections and at several time points after the injections (in general, every 15 min), aliquots of 150 microliter blood were removed from the femoral vein. Whole blood and plasma samples were deproteinized by adding an equal volume of ice-cold 1M perchloric acid (PCA) and then centrifuging at 16000×g for 10 min at 4^0C. The pellet was discarded and supernatant collected, adjusted to pH 7.2 with 2M K_2CO_3 and, if needed, stored at -20^0C for later analysis. In vitro experiments were carried out as described in Yin et al. (2002).

When glutamate concentration in the cell compartment was analyzed, blood was centrifuged at 1,300×g for 7 min at 4^0C and the cell pellet (erythrocytes, granulocytes, leukocytes, platelets) was resuspended in double distilled water up to a final volume of 100microliter and an identical volume of 1M PCA added and treated as above.

Glutamate concentration was measured in the supernatant using the fluorometric method of Graham and Aprison (1966). A 2microliter aliquot from PCA supernatant was added to 480microliter HG buffer containing 15 U of glutamate dehydrogenase in 0.2mM NAD, 1M glycine, 2.4% hydrazine hydrate adjusted to pH 8.6 with 1N H_2SO_4 . After incubation for 30-45 min at room temperature, the fluorescence was measured at 460 nm after excitation at 350 nm. A glutamate standard curve was established with concentrations ranging from 0-6 micromolar. All determinations were done in duplicates. The results are expressed as mean.+-SD.

Blood Pyruvate concentration was measured using the Sigma Diagnostics Pyruvate reagents and the Sigma protocole. The procedure utilizes lactate

dehydrogenase which in the presence of excess NADH catalyzes the almost complete conversion of pyruvate into lactate with a concomitant production of NAD. The reduction of absorbance at 340 nm due to the oxidation of NADH into NAD becomes the measure of the amount of pyruvate originally present.

Blood oxaloacetate concentration was measured using the same procedure as for pyruvate but using malate dehydrogenase which catalyses the conversion of oxaloacetate into malate along with the oxidation of NADH into NAD.

Results

Taking into consideration that the rat blood volume is about 5.5 to 7 ml per 100 g body weight (Van Dongen et al. 1990; Waynforth and Flecknell, 1992), we first tested the effects on rat Glu levels of a single intravenous injection of a mixed solution in PBS of pyruvate and oxaloacetate, each at a dose of 30 micromolaroles (2 ml of a 15 mM solution). Our aim was to reach a final blood concentration of 1mM for both pyruvate and oxaloacetate, since these were the optimal concentrations found for an effective decrease of blood Glu in in vitro experiments (Yin et al. 2002). However, no significant effects on blood Glu were observed even when the intravenously injected doses were increased to 200 micromolaroles (data not shown) or when up to 1mmole was administered either subcutaneously or intraperitoneally.

Figure 1 shows that when four successive intravenous injections of pyruvate and oxaloacetate, each at a dose of 30 micromolaroles, were performed (at 15 min intervals), in the absence or presence of glutamate dehydrogenase (GDH) activators (3 micromolaroles each) of Leucine, NAD and ADP, a 10-20 % decrease of blood Glu levels was observed. Under parallel in vitro conditions, a 40-60% decrease of blood Glu levels took place. In both cases, the decrease was transient and an increase of blood Glu levels took place soon after the third or fourth injection.

Figure 2 illustrates the effects of pyruvate and oxaloacetate administered through an intravenous cathether at a rate of 50 micromolaroles/ min for a duration of 30 min. The blood levels of Glu, pyruvate and oxaloacetate were monitored in

parallel. It can be seen that a significant build up of both pyruvate and oxaloacetate takes place after 15 min and is accompanied by a marked decrease of blood Glu. However, as soon as the administration of the GOT/GPT co-substrates is stopped, their blood concentration decrease and that of Glu increases concomitantly.

Figure 3 shows that when the levels of Glu are monitored for about 200 min after the completion of the infusion of pyruvate and oxaloacetate, a clear overshoot of Glu above blood basal levels is observed, suggesting that the increase of Glu levels is not only due to the reversal of the GOT/GPT reactions (as a result of the build-up of the enzymatic products, 2-ketoglutarate, alanine and aspartate) but to additional compensatory processes. It should be mentioned that the administration of pyruvate and oxaloacetate was not accompanied by changes in the rat pulse rate or rectal temperature and but produced a diuretic effect likely to be due to an increase of blood Na^+ ion concentration since pyruvate and oxaloacetate were injected as sodium salts.

As described in the paper of Yin et al (2002), the in vitro addition to blood of lipoamide or of thiaminepyrophosphate, two cofactors of the 2-ketoglutarate dehydrogenase, limits by about 20% the GOT and GPT back reactions by decreasing the availability of 2-ketoglutarate as the latter is converted into succinyl CoA. To determine whether similar effects take place in vivo, we carried out a 30 min long intravenous injection of pyruvate and oxaloacetate (7.5 micromolaroles/ min) together with lipoamide (75 nmoles/min) or thiaminepyrosphate (5 nmole/min). In these experiments, Glu levels were monitored in blood as well as in its two compartments, plasma and cells. When analyzing blood, no significant differences were observed from those presented in Figure 3 indicating that a saturating effect of pyruvate and oxaloacetate is reached with the administration of 7.5 micromolaroles/ min. Moreover, at the doses tested, lipoamide and thiaminepyrophosphate had no significant contributions and did not increase the pyruvate and oxaloacetate-mediated Glu conversion in vivo. Analysis of the Glu levels in the respective blood plasma and cell pools shows that the initial rates) of Glu conversion (defined as the % Glu

conversion in the first 15 minutes) in the cell pool (3.3%/min) differs significantly from that in plasma (0.6%/mn). Moreover, though the extent (60%) and rate of Glu conversion in the cell pool measured in vivo are similar to those measured in vitro (Yin et al. 2002), the extent of Glu conversion measured for plasma in vivo is about half that measured in vitro. The latter results suggest that the decrease in plasma Glu levels produced by the activation of GOT/GPT, causes various organs to release their Glu content into plasma to adjust to the new situation.

Discussion

Duplication in vivo of the in vitro conditions leading to a decrease in blood Glu levels i.e activation of GPT/GOT upon injection of 1mM pyruvate/oxaloacetate failed to achieve any significant decrease of blood Glu levels. This was quite expected since in vitro conditions are obviously not affected by pharmacokinetic parameters such as elimination rate constants, volume of distribution or renal clearance from plasma. To overcome the latter processes, higher concentrations of pyruvate/oxaloacetate were needed to be injected in blood in order to build up effective concentrations allowing the activation of GPT and GOT. Thus when 1.5 mmoles of pyruvate/oxaloacetate were continuously injected intravenously over a period of 30 min, effective blood concentrations of about 0.5 mM were reached which led to a decrease of blood Glu levels. These concentrations are within the range of the GPT/GOT apparent Km values for pyruvate/oxaloacetate measured upon incubation of blood under in vitro conditions (Yin et al. 2002). Though we did not explore systematically the effective range of pyruvate/oxaloacetate concentrations in vivo leading to a decrease of blood Glu levels, the results presented in Figure 4 suggest that the administration of 0.225 mmoles of pyruvate/oxaloacetate is as effective as 1.5 mmoles.

It is of importance to emphasize the point that extremely high doses of pyruvate/ oxaloacetate had to be administered in order to reach effective concentrations in blood and obtain a significant decrease of Glu levels. This is due to

the fact that both pyruvate and oxaloacetate have very unfavorable pharmacokinetic parameters. They have a very large distribution volume and are in addition avidly pumped and metabolized in all encountered organs. Thus, pyruvate/oxaloacetate have to be infused at slow rates and high doses compatible both with the build up of effective blood concentrations and the prevention of hemolysis. At present, the administration of pyruvate/oxaloacetate can be used in experimental situations but is clearly unacceptable in any therapeutic context. One possible future solution would be the in vivo administration of pyruvate/oxaloacetate together with GOT / GPT species displaying increased affinity for these co-substrates. Such improved versions of GOT or GPT could possibly be generated by in vitro evolution.

It is interesting to note that the blood cellular pool is particularly sensitive to the presence of pyruvate /oxaloacetate: it looses its Glu content as soon as pyruvate and oxaloacetate reach an effective concentration in blood, and starts regaining it as soon as the administration of pyruvate/oxaloacetate stops.

The rather rapid recovery of Glu in the blood cellular pool can be attributed to several factors that probably act in synergy:

1) It could be due in part to the reversal of the GOT/GPT enzymatic reactions that cause the synthesis of Glu from 2-ketoglutarate, alanine and aspartate that accumulate during the forward reactions.

2) An uptake of Glu from plasma or the adsorption of Glu onto blood cell membranes (Pico et al. 1995) could contribute to the recovery of Glu content in the blood cellular pool since the latter takes place concomitantly with a decrease of plasma Glu.

3) An uptake of plasma glutamine followed by its hydrolysis into Glu could also take place as a result of the activation of blood cell glutaminases which are normally inhibited by Glu (Roberg et al. 2000). Thus, a temporary decrease in the availability of Glu could be compensated by the increased enzymatic hydrolysis of Gln which is present in plasma and cells at concentrations about four to five times that of Glu. Such compensatory effect mediated by Gln and glutaminases is expected

to be similar when blood Glu levels are followed under in vitro or in vivo conditions since Gln and the glutaminases are present in both.

However, it is unlikely that Glu has a significant contribution since the kinetics of recovery of Glu levels in both plasma and blood cell compartment is much slower under in vitro conditions than in vivo.

4) Another factor is an increased tissue-to-blood Glu efflux. Indeed, one can expect the transiently increased plasma-to-tissue Glu gradient to facilitate the efflux of Glu from tissue cytoplasmic pools into plasma. Such in efflux in turn will cause an activation of tissue glutaminases and a further production of Glu.

Which tissues could contribute to this Glu enhanced influx into plasma? A distinction has been made between organs expressing a net Glu release such as the liver, or a net Glu uptake such as the intestine, lungs and muscles and organs such as the kidney and brain with no net Glu removal or release (Hediger and Welbourne, 1999). However, this classification may be different under conditions of decreased plasma Glu levels. Muscle for instances has a very large pool of free Glu (Graham et al. 2000) that could possibly be released under conditions of low plasma Glu.

Of all the above mentioned factors, the only one likely to contribute to the large overshoot of blood Glu levels above the control levels (Figure 3) is the increased organs-to-blood Glu efflux. Such Glu movements could also account for the fact that the kinetics of evolution of plasma Glu during the intravenous administration of pyruvate/oxaloacetate is slower than that of Glu in the blood cell compartments. Indeed, if the decrease of plasma Glu provokes a concomitant increased Glu efflux from tissues, one expects such compensatory efflux to manifest itself by the limited extent and slow kinetics of plasma Glu changes.

In summary, blood Glu levels are decreased as a result of the activation of the blood resident enzymes GOT and GPT by the Glu co-substrates oxaloacetate and pyruvate, but the effect is transient and rapidly compensated most probably by an increased Glu efflux to blood from Glu-containing organs. It remains to be determined whether the latter process includes a significant contribution from brain.

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Legends of Figures

Figure 1

Evolution of blood glutamate levels in vivo (black symbols) and in vitro (open symbols) upon repeated administrations (every 15 min from t=0 up to t=45 min; black arrows) of 200 microliter PBS containing pyruvate/oxaloacetate (30 micromolaroles each) in the presence (circles) or absence (black diamonds and open squares) of NAD and ADP (3 micromolaroles each). Symbols show averages of two Glu level measurements. One representative experiment out of 4 performed is shown.

Figure 2

Evolution of blood glutamate levels in vivo (black squares) upon intravenous administration for a duration of 30 min (black bar) of pyruvate and oxaloacetate (50 micromolaroles each / min). The blood levels of pyruvate (circles) and oxaloacetate (triangles) were monitored in parallel. Symbols show averages of two determinations.

Figure 3

Evolution of blood glutamate levels in vivo upon intravenous administration for a duration of 30 min (black bar) of pyruvate and oxaloacetate (50 micromolaroles each / min). Symbols show averages of two Glu determinations from 7 experiments.

Figure 4

Evolution of blood glutamate levels in vivo upon intravenous administration, for a duration of 30 min (black bar), of a PBS solution containing 150 mM pyruvate, 150 mM oxaloacetate and 1.5 mM lipoamide administered at a rate of 50 microliter/min. The levels of glutamate in blood (circles), blood cell compartment (triangles) and plasma (squares) are presented. Symbols show averages of two Glu determinations. One representative experiment out of 2 performed is shown.

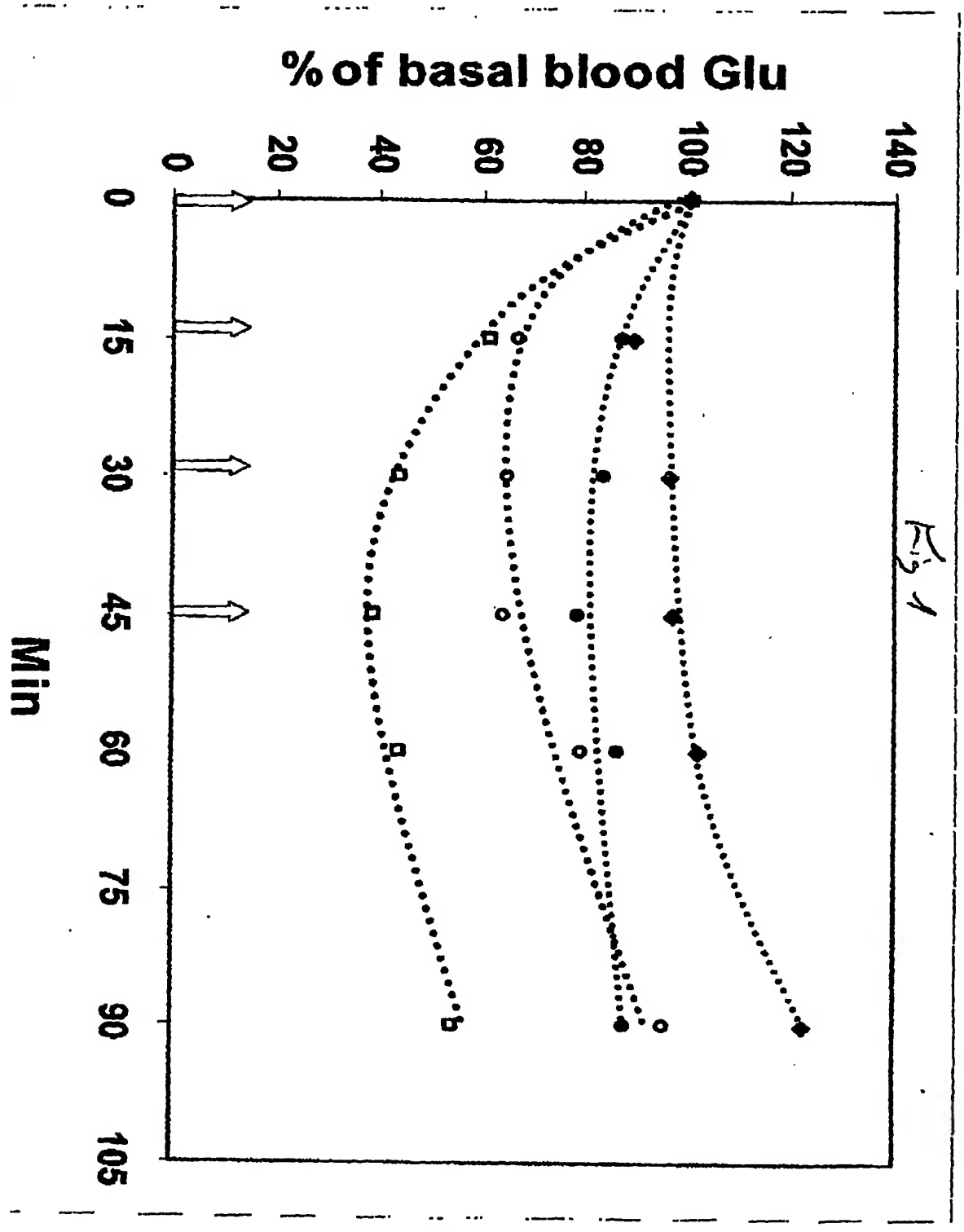
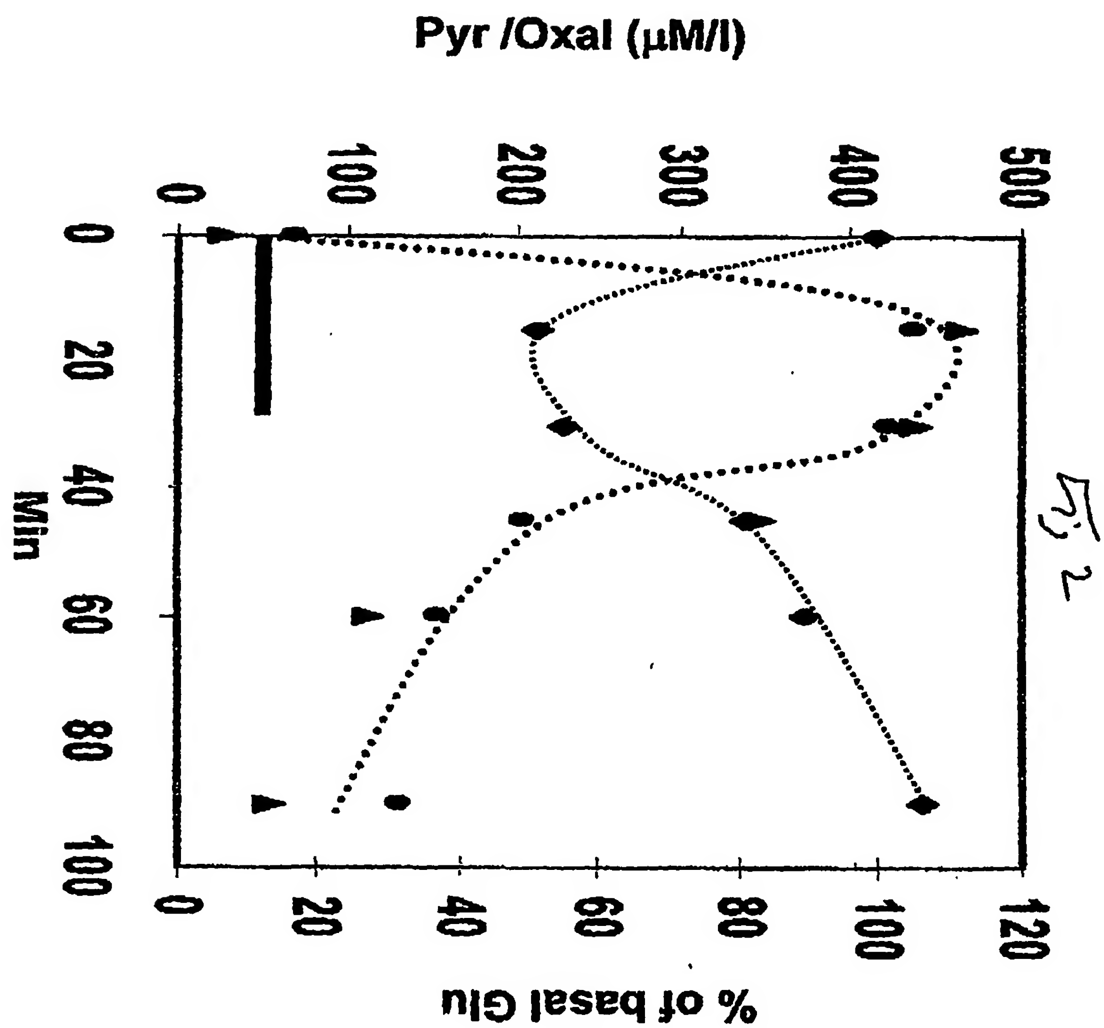


Fig 1


Pyr /Oxal (µM/l)
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100
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120

Fig 2

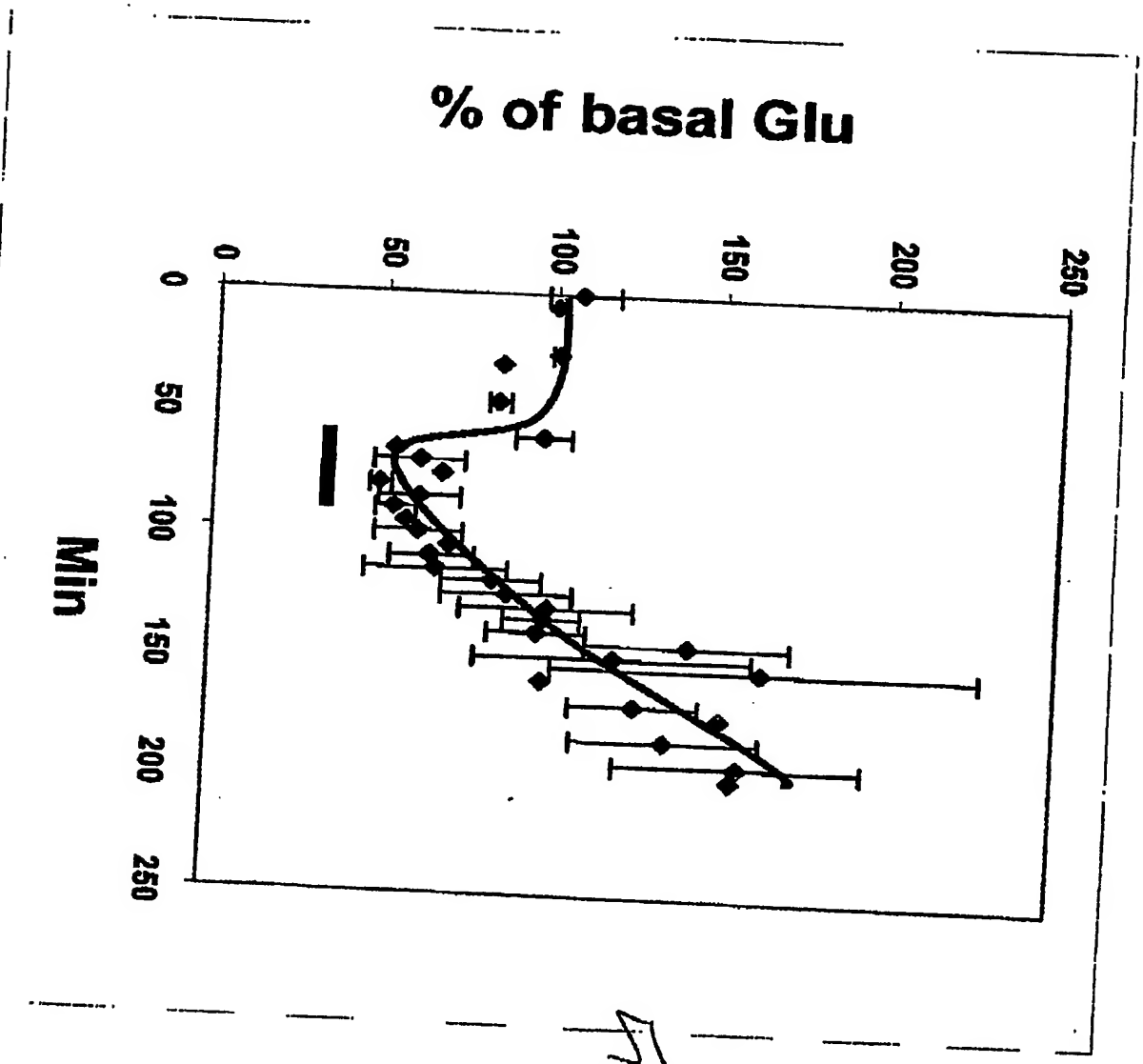

Fig. 3

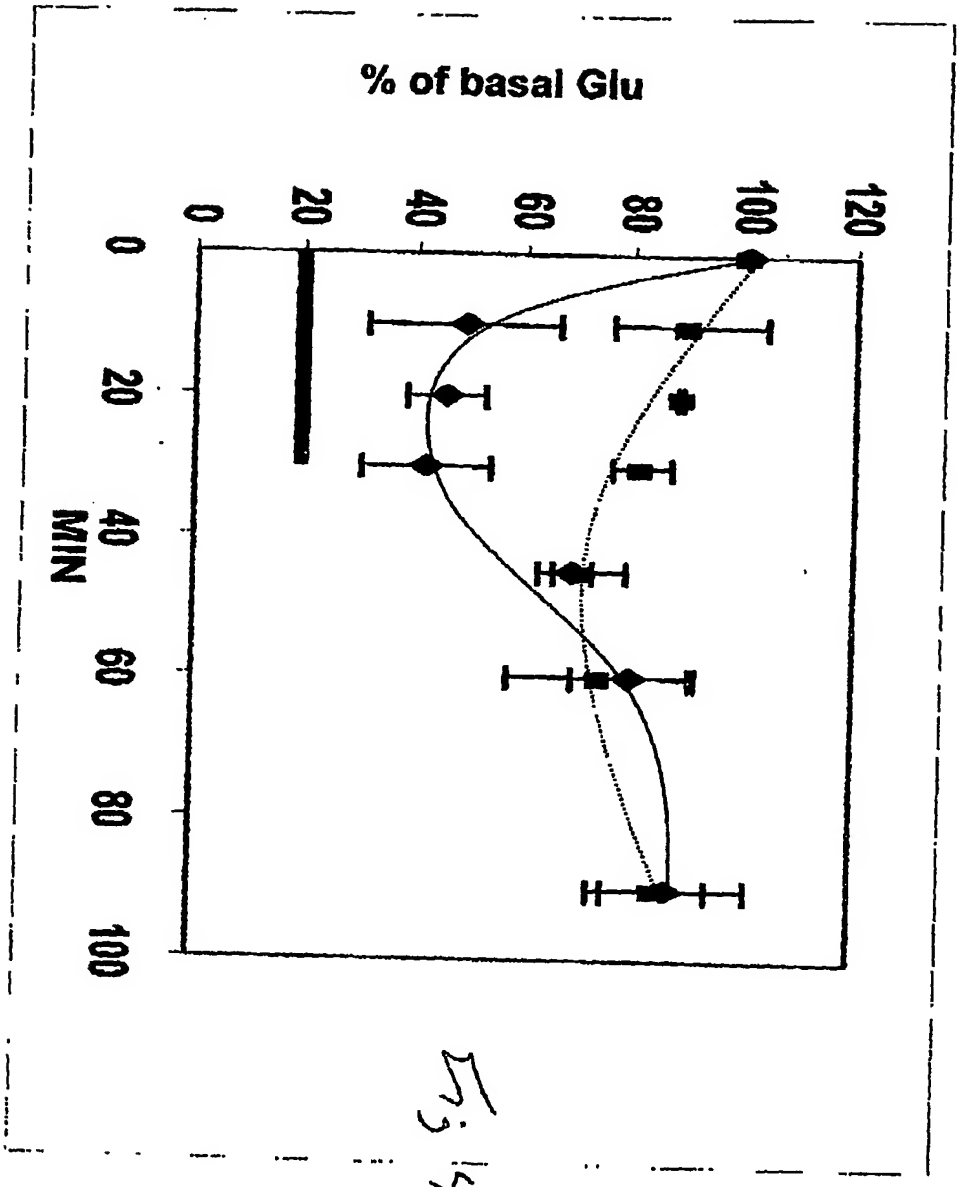

Fig 14

C. GLUTAMATE UPTAKE INTO THE BLOOD CELL COMPARTMENT AND ITS CONTRIBUTION TO THE DEPLETION OF PLASMA GLUTAMATE DURING BLOOD EXCHANGE

Abstract

Several intractable brain pathological conditions are characterized by the presence of excess glutamate in brain interstitial fluid. In order to increase the driving force for an enhanced brain-to-blood efflux of glutamate, we have investigated here the glutamate-pumping ability of glutamate-depleted blood cells. Two components were identified: an instantaneous uptake process that was attributed to an adsorption of glutamate on blood cell membranes and a slow developing uptake attributed to glutamate diffusion into cells. When glutamate-depleted blood cells resuspended in 6% hetastarch were injected into a recipient rat using a blood exchange protocol, a transient decrease of plasma glutamate was observed. Upon isovolemic haemodilution with 6% hetastarch, the kinetics of the decrease of the hematocrit was faster than that of blood glutamate levels. This observation was interpreted as suggestive of a hemodilution related compensatory glutamate efflux into blood from various organs including brain.

Introduction

Several chronic and acute degenerative conditions of the brain such as stroke (Castillo et al. 1996), head trauma (Zauner et al. 1996), amyotrophic lateral sclerosis (Shaw et al. 1995) and AIDS dementia (Ferrarese et al. 2001) are characterized by the presence of excess levels of the excitatory neurotransmitter Glutamate (Glu) in the interstitial fluid (ISF) and cerebrospinal fluid (CSF). Since at such levels, Glu can exert neurotoxic properties and kill neurons, neuroprotective strategies are relentlessly being evaluated with the hope of finding ways to neutralize the deleterious effects of Glu and its ensuing neurological deficits.

In this context, we have made the hypothesis that brain neuroprotection could be achieved by accelerating the still little known process of brain to blood Glu efflux. Since this efflux takes place against a ISF/CSF to blood Glu concentration gradient, our assumption is that the brain to blood Glu efflux should be significantly facilitated upon a decrease of Glu concentration in blood.

In previous papers (Yin et al. 2002; Gottlieb et al. 2002), we have shown that such a decrease takes place, both in vitro and in vivo, upon activation of the blood resident enzymes Glu-pyruvate transaminase (GPT) and Glu-oxaloacetate transaminase (GOT) with the Glu co-substrates pyruvate and oxaloacetate. Repeated additions to blood or intravenous administration of pyruvate and oxaloacetate cause a decrease of Glu both in plasma as well as in the blood cell compartment in which 80% of blood Glu resides.

On the above premises, we reasoned that if the Glu-depleted blood cell compartment would be able to rapidly pump plasma Glu towards the original cell/plasma Glu concentration ratio of ~4, it could provide a novel blood exchange strategy of blood Glu reduction possibly applicable in emergency cases such as stroke and head trauma in which the observed acute increase of Glu in the ISF/CSF is thought to initiate the neurodegenerative process.

Previous studies carried out with rat and human blood have emphasized the relative impermeability of erythrocytes to extracellular Glu (Young et al. 1980; Pico et al. 1992; Culliford et al. 1995) but those were carried out in the presence of an unfavorable Glu concentration gradient. It was thus of interest to investigate the Glu transport properties of Glu-depleted blood cells and their contribution to the reduction of plasma Glu during blood exchange. In addition, this study was expected to shed some light on the still unexplained origin of the large blood Glu intracellular pool.

Materials and Methods

Glu dehydrogenase was from Roche; Glu-pyruvate transaminase was from Sigma. All chemicals were purchased from Sigma unless noted otherwise.

Blood was collected retroorbitally from 200-250 g SPD rats anaesthesized intraperitoneally with 40 mg ketamine and 5 mg xylazine/kg body weight. Human blood was obtained from a blood bank. Fresh rat blood was incubated at 37°C in the presence of pyruvate, oxalacetate or a combination of both. Every 15 min, when needed, blood was supplemented with these substrates (freshly prepared 100 mM stock solutions) to obtain a final concentration of 1mM. When glutamate incorporation was studied in glutamate-depleted cells, the latter were washed and resuspended in Ringer-Hepes buffer containing 2.75 mM glucose and glutamate at various concentrations from 0.1 to 1 mM. When blood compartments were analyzed, the blood cell compartment was defined as the blood cells present in the pellet obtained by centrifugation of blood at 9000g.min while plasma was defined as the supernatant resulting from that centrifugation. For Glutamate analysis, blood aliquots of 150microliter were removed at each time point and centrifuged at 1,300×g for 7 min. The volume of supernatant (plasma) was measured and an identical volume of 1M PCA (perchloric acid) was added to precipitate proteins. The cell pellet was resuspended in double distilled water up to a final volume of 150microliter and an identical volume of 1M PCA was added. Both plasma and cell PCA-precipitated fractions were centrifuged at 16,000×g for 10 min and the pellet discarded.

Glu concentration was measured in the supernatant using the fluorometric method of Graham and Aprison (1966). A 20microliter aliquot from PCA supernatant was added to 480microliter HG buffer containing 15 U of Glu dehydrogenase in 0.2mM NAD, 1M glycine, 2.4% hydrazine hydrate adjusted to pH 8.6 with 1N H_2SO_4 . After incubation for 30-45 min at room temperature, the fluorescence was measured at 460 nm after excitation at 350 nm. A Glu standard curve was established with concentrations ranging from 150 microliter. All determinations were done in duplicates. The results are expressed as mean.+-SD.

Blood exchange: The blood transfusion experiments were carried out with SPD rats (250- 300g). The blood donor rat was anaesthetized with 60 mg/kg Pentobarbital, its chest opened and blood was withdrawn from the heart and collected into a tube containing heparin (0.8 mg/ml of collected blood). The blood was incubated at 37°C with oxaloacetate and Pyruvate added every 10 min to a final concentration 1 mM. After 40 min, the blood was centrifuged at 4000 rpm/10 min and the plasma withdrawn. The pellet was resuspended to the original blood volume into a 6% solution of hetastarch in 0.9% NaCl. Blood exchange into an anaesthetized recipient rat was performed by placing a polyethylene cannula (PE 10) in the femoral vein for blood infusion and a polyethylene cannula (PE 10) in the femoral artery for blood withdrawal.

Blood was transfused at a rate 0.75 ml/min using a peristaltic pump while arterial blood was withdrawn at the same rate of 0.75 ml/min with another peristaltic pump. .

Glu levels in the donor rat blood were monitored after blood withdrawal and during the in vitro incubation with oxaloacetate and pyruvate. Glu levels in the recipient rat were monitored by removal of 200 microliter blood aliquots from the femoral vein or the femoral artery twice before the beginning of the blood exchange, immediately after the end of the exchange, and 15, 30, 60, 90 min postexchange. The total blood exchange times varied between 12-17 min.

Isovolemic haemodilution was carried out over a period of 25 min by substituting each ml of blood that was removed by an equivalent volume of 6% Hetastarch. Glu levels in the recipient rat were monitored in 100 microliter aliquots from each ml of blood removed. The remaining 900 microliter were centrifuged at 4000 rpm/10 min and the respective volumes of pellet and supernatant were measured and their ratio was defined as the haematocrit.

Results

Figure 1 illustrates first the decrease of Glu levels in the blood cell compartment obtained in vitro upon additions of pyruvate/oxaloacetate carried out every fifteen minutes for a total duration of 90 min. It then shows the increase of Glu levels resulting from the exposure of the cell compartment to various Glu concentrations from 0.1 to 1 mM and monitored every fifteen minutes for a total duration of 60 min. It can readily be seen that as soon as the cells are exposed to Glu, an "instantaneous" incorporation of Glu takes place followed by a slow developing increase that tends to saturate with time. These processes can be entirely attributed to erythrocytes since purified neutrophils, lymphocytes or platelets did not display any Glu uptake (data not shown).

Plotting the "instantaneous" cell Glu increase as a function of Glu concentration (Figure2) shows that this process has an apparent Michaelian behavior and saturates. Analysis of the curve provides an apparent Km of 488 ±172. micromolar and a Bmax of 70±11 nmol Glu/ml of cell suspension. Figure 3 presents a plot of the total Glu increase that took place during the entire 60 min duration of the uptake experiment, as a function of Glu concentration. This "slow" increase in Glu cell content appears linear with the increase of external Glu concentration and presents no indication for saturation. It could thus be attributed to a diffusive process.

It can be calculated from the curves presented in Figures 2 and 3 that for Glu concentrations below 600 micromolar, the capacity of the "instantaneous process is about 2.4 times larger than the slow uptake process. Thus, the "instantaneous" uptake process that saturates and the "slow" uptake processes that does not, have different Glu uptake capacities suggesting that they correspond to separate compartments.

To further examine the nature of the "instantaneous" and "slow" increases of Glu cell content, we followed the fate of Glu when cells incubated for 60 min in 1mM Glu (as in Figure 1) were exposed to a Glu free solution. Figure 4 shows the evolution of Glu levels in cells and supernatants as a function of time. It can be seen

that two processes affect the dynamics of Glu: an "instantaneous" one that produces a decrease of cell-associated Glu by about 30% and an equivalent increase of Glu in the cell supernatant, and a slow developing one which produces a decrease with time of the cell Glu content at a rate about three times faster than that of the parallel Glu level increase in the cell supernatant. The simplest explanation for this difference in rates is that the decrease of cell Glu content is not only due to the diffusion of Glu down the concentration gradient but also to the intracellular utilization of Glu as a metabolite. To support this interpretation, we carried out the above experiments at two temperatures: the slow decrease of cell Glu content was found to be about three times larger at 37°C than that at 20°C while the "instantaneous" uptake was greater at 37°C than at 20°C by a factor of 1.8 (data not shown).

The in vitro ability of Glu-depleted blood cells to take up Glu from plasma was investigated and the results presented in Figure 5. It can be seen that the exposure of Glu-depleted blood cells to plasma causes an "instantaneous" decrease of plasma Glu by about 30% with a parallel increase of the blood cell Glu content which is sustained for 60 min. Moreover, the "slow" cell Glu uptake component observed when cells are exposed to the buffers used in Figures 1 and 4, appears to be reduced when cells are exposed to plasma in line with the Km value estimated in Figure 2.

To evaluate the in vivo ability of Glu-depleted blood cells to take up Glu from plasma, we carried out blood exchange experiments in which blood was removed from a recipient rat and exchanged with Glu-depleted blood cells suspended in 6% Hetastarch. Under the conditions of the experiment presented in Figure 6, 8 .5 ml of a blood cell suspension with cells harboring 30% of the Glu levels present in the host blood cell compartment were administered in 13 minutes and exchanged for an equivalent blood volume corresponding to 40% of the total host blood volume. Glu levels in the host rat blood were monitored both in the plasma and cell compartments. It should be noticed that soon after the termination of the blood exchange procedure, the Glu losses in plasma recovered with a kinetics

very similar to that observed when an intravenous administration of pyruvate and oxaloacetate was carried out (Gottlieb et al. 2002).

Analysis of Figure 7 shows that the Glu content of the host cell compartment changes by up to 20% changes, in contrast to the 35% changes in plasma. The host plasma is indeed expected to loose its Glu content not only because of the Glu pumping ability of the donor blood cells but also because of its dilution with the Hetastarch vehicle. This issue was investigated and Figure 7 shows the evolution of the hematocrit and of the blood Glu concentration in a rat submitted to isovolemic haemodilution by substituting each ml of blood that was removed by an equivalent volume of 6% Hetastarch. Interestingly, the evolution of the hematocrit is much steeper than that of blood Glu suggesting that an increased efflux of Glu from various organs into blood compensate for the blood Glu dilution.

Discussion

In the present study, we tested the ability of Glu-depleted blood cells to pump Glu from plasma in the hope that it could provide a novel blood exchange strategy of blood Glu reduction, and hence of brain ISF/CSF Glu levels (Gottlieb and Teichberg, 2202), possibly applicable in emergency cases such as stroke and head trauma in which the observed acute increase of Glu in the ISF/CSF is thought to initiate the neurodegenerative process.

Erythrocytes which compose the vast majority of blood cells are notoriously poorly permeable to Glu. This applies to rat and human erythrocytes (Pico et al. 1992; Ellory et al. 1983; Darmaun et al. 1986) but not to dog red blood cells (Ellory et al. 1983). However, the low permeability of rat and human erythrocytes was deduced on the basis of experiments in which the transport of Glu was measured in the presence of an unfavorable Glu concentration gradient. It was therefore of interest to revisit this issue under conditions of a favorable Glu concentration gradient. Under these conditions, two Glu compartments could be distinguished: an "instantaneous" process that saturates with increasing Glu concentrations and a slow

developing process that does not. The latter, but not the former, have been described by Pico et al. (1992) and ascribed to diffusion because the uptake does not display saturation kinetics. The Glu transport process measured here (K= 0.144±0.02 /hour (data not shown)) is slightly slower than that (K=0.218/hour) studied by Pico et al. (1992).

The origin of the "instantaneous" process is not obvious at first. It cannot originate from platelets or lymphocytes because it is not affected by the removal of these cells (data not shown). It can also not be an artifact due to trapping of Glu within the cell pellet because the solute trapping, measured with [^{14}C]-sucrose (data not shown), did not exceed 6% of the input and because the trapping process is not expected to reach saturation. The most likely explanation for the "instantaneous" process is that it corresponds to the pool of Glu that is adsorbed by blood cell membranes (Pico et al. 1995). Its low apparent affinity (Km = 0.48 mM) is in line with the fact that it can rapidly dissociate and be washed away (see Figures 5 and 6). Its capacity of about 30% of the total Glu associated with blood cells is larger than the value of 18% reported by Pico et a (1995) but this discrepancy can be accounted on methodological grounds.

In any event, the Glu uptake studied here with Glu-depleted erythrocytes displays a behavior similar to that of Glu-containing blood cells (Pico et al. 1995) as expected for a purely diffusive process. However, the low Glu permeability of red blood cells raises difficulties in explaining the high red blood cells to plasma Glu concentration ratio which in humans is around 8 and 3 in rats. Such a ratio would be simple to explain would erythrocytes like neurons be able to transport and concentrate Glu effectively. As this is not the case, plasma Glu is unlikely to be the source of intraerythrocytic Glu, and a rise in plasma Glu would result only in a minimal rise of intracellular Glu in erythrocytes. Glutamine which is about 20 times more abundant than Glu in plasma and is easily transported across cell membranes, has been assumed to serve as the intracellular source of Glu.[53] However, since the conversion of Gln to Glu depends on sufficient glutaminase, a mitochondrial

enzyme, and because mature erythrocytes do not contain mitochondria, intracellular Glu is unlikely to depend on the high permeability and availability of Gln.

It has been suggested that red blood cells act as carriers in inter-organ amino-acid transport (Felig et al. 1975). However, it is unlikely that the erythrocyte intracellular Glu pool plays an important role in the highly dynamic inter-organ Glu transport since the Glu efflux is too slow to account for the observed Glu arterio-venous differences (Heitman and Bergman, 1980; Darmaun et al. 1986). The latter can most probably be ascribed to the erythrocyte extracellular Glu pool that can readily release and adsorb Glu.

Putting the Glu binding and transport properties of red blood cells in the context of a proposed strategy of blood exchange for achieving a plasma Glu reduction possibly applicable in emergency cases such as stroke and head trauma, it is clear that Glu-depleted red blood cells have a limited but non negligible ability to reduce plasma GLU. The latter is reduced by about 30% within 15 min and does not recover its original value for at least 90 min. This decrease of Glu plasma can be accounted not only to the "instantaneous" Glu binding to red blood cells but also to the plasma dilution brought about by the blood exchange with a Hetastarch solution.

The slow kinetics of plasma Glu recovery which is similar to that observed upon intravenous administration of pyruvate and oxaloacetate can be most probably accounted by an increased organs-to-blood Glu efflux in response to the decrease of plasma Glu mediated by blood exchange or hemodilution. Many organs including brain can possibly contribute to this process. It is of interest to mention in the latter context that hemodilution with 10% hetastarch following closed-head trauma in rats was found to be beneficial and decreased brain tissue necrosis volume while improving the neurological severity score (Chorny et al. 1999). Would the latter observation be due to an enhanced brain-to-blood Glu efflux, a blood exchange strategy could be proposed in the emergency conditions such as stroke and head trauma in which a reduction of CSF/ISF Glu would be desired.

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Legends of Figures

Figure 1

Glutamate uptake/adhesion to glutamate-depleted rat blood cells. Evolution of Glu levels in the rat blood cell compartment in vitro upon additions of pyruvate and oxaloacetate to a final concentration of 1 mM repeated every fifteen minutes for a total duration of 90 min (filled circles). Cells were then washed and resuspended in Ringer Hepes buffer containing Glu at various concentrations. The incorporation of Glu in the blood cell compartment was monitored every fifteen minutes for a total duration of 60 min. Symbols: filled diamonds: Ringer Hepes buffer; grey squares: 0.1 mM Glu; grey crosses: 0.2 mM Glu; grey circles: 0.4 mM Glu; triangles: 0.6 mM Glu; greyer circles: 0.8 mM Glu; grey diamonds. 1 mM Glu. Each point represents the average of at least two Glu determinations.

Figure 2

Concentration dependence of the "instantaneous" Glu uptake/adhesion to glutamate-depleted rat blood cells. Data were derived from figure 1. The process

displays a michaelian behavior with a Km = 488 ±172 micromolar and Bmax = 70±11 nmol Glu/ml of cell suspension.

Figure 3

Concentration dependence of the "slow" Glu uptake/adhesion process to glutamate-depleted rat blood cells. Data were derived from figure 1. The process is linear and displays a correlation coefficient $R^2 = 0.97$.

Figure 4

Kinetics of Glu release from cells loaded with 1 mM Glu and placed in Ringer Hepes solution devoid of glutamate. Filled circles: evolution of Glu levels in the blood cell compartment. Filled squares: evolution of Glu levels in the extracellular solution. Arrows indicate the extent of the "instantaneous" Glu release (downward) from cells and of the "instantaneous" Glu appearance in extracellular solution (upward). Each point represents the average of at least two Glu determinations.

Figure 5

Plasma glutamate uptake/adhesion to glutamate-depleted rat blood cells. Evolution of Glu levels in the rat blood cell compartment in vitro upon additions of pyruvate and oxaloacetate to a final concentration of 1 mM repeated every fifteen minutes for a total duration of 90 min (filled squares). Cells were then washed and resuspended in plasma. The levels of Glu in the blood cell compartment (black squares) and plasma (black circles) were monitored every fifteen minutes for a total duration of 60 min. Each point represents the average of at least two Glu determinations.

Figure 6

Evolution of blood glutamate levels in vivo following blood exchange. Blood (8.5 ml) was removed from a recipient rat and exchanged over 13 min with 8.5 ml

of Glu-depleted blood cells suspended in 6% Hetastarch. Depletion of Glu in the rat blood donor was achieved by incubation in vitro with pyruvate and oxaloacetate at a final concentration of 1 mM added every fifteen minutes for a total duration of 60 min. Under these conditions a Glu depletion of 70% was achieved. The amount of blood exchanged corresponds to 40% of the total host blood volume. The levels of glutamate in the blood cell compartment (diamonds) and plasma (squares) are presented. Symbols show averages of two Glu determinations. One representative experiment out of 3 performed is shown.

Figure 7

Evolution of blood glutamate levels in vivo following isovolemic hemodilution with 6% hetastarch. Blood aliquots of 1 ml were removed every 3 min from a recipient rat and exchanged with a 6% Hetastarch solution in PBS. Depletion of blood Glu (squares) and the hematocrit (triangles) was monitored in each aliquots. One representative experiment out of 3 performed is shown.

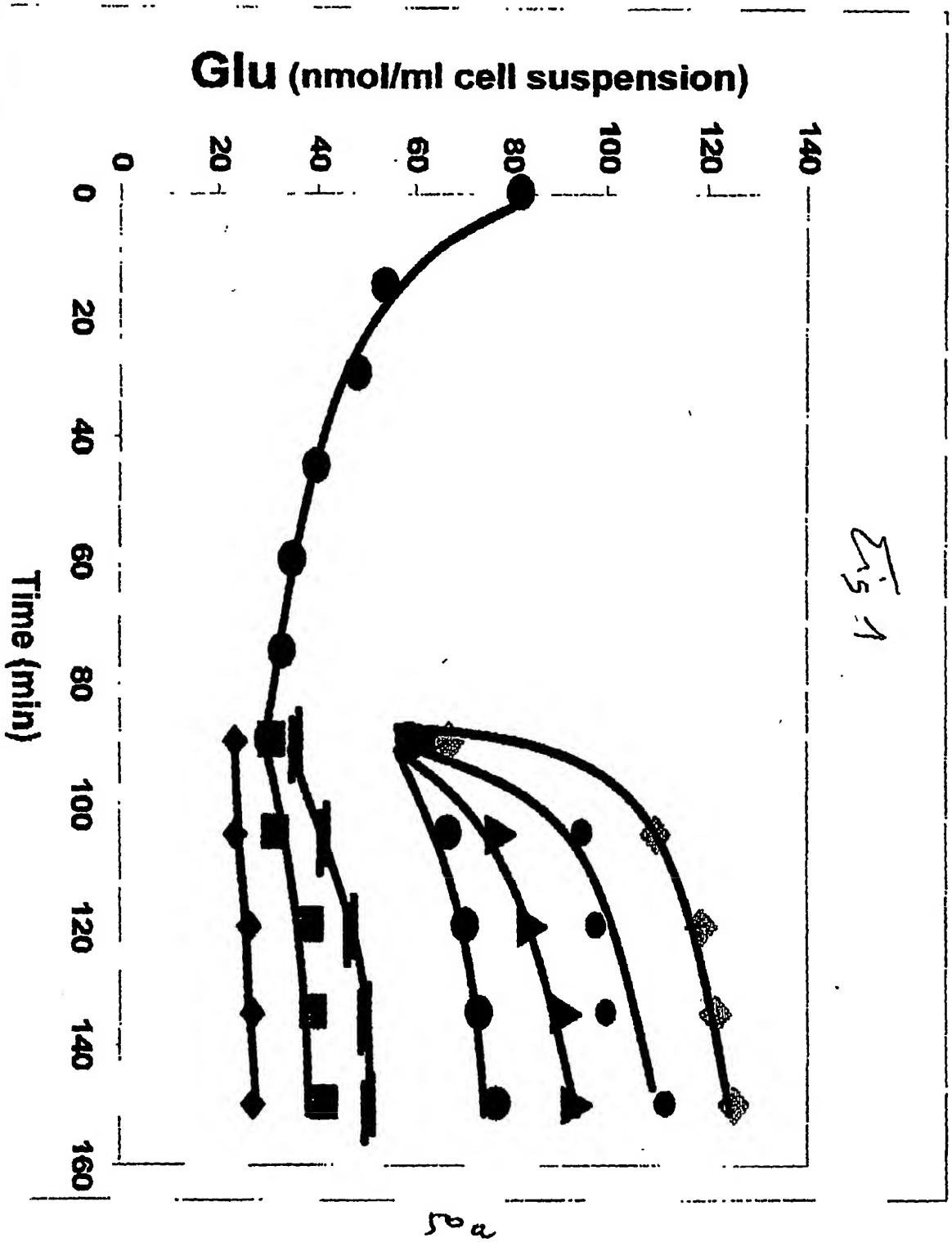
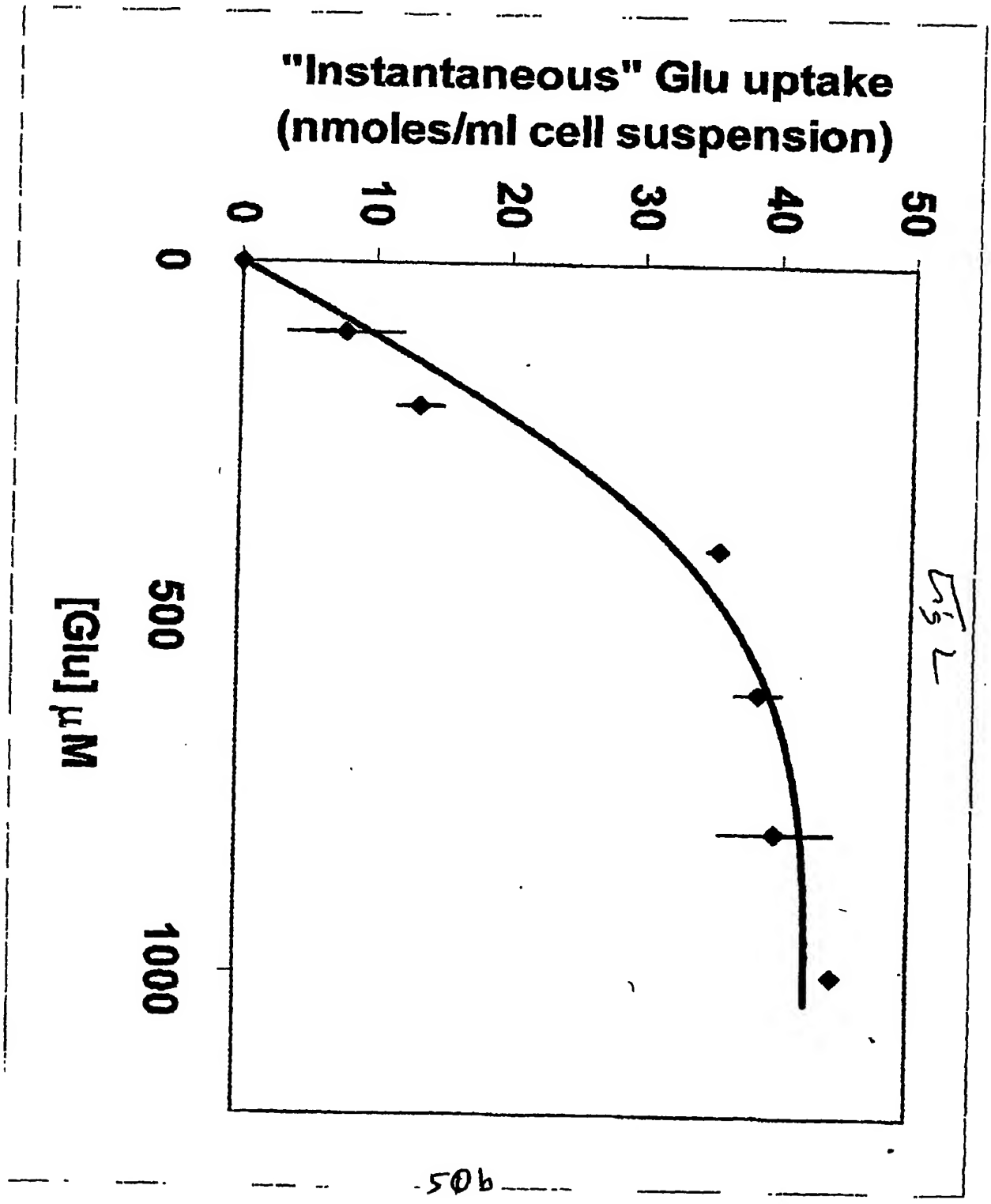
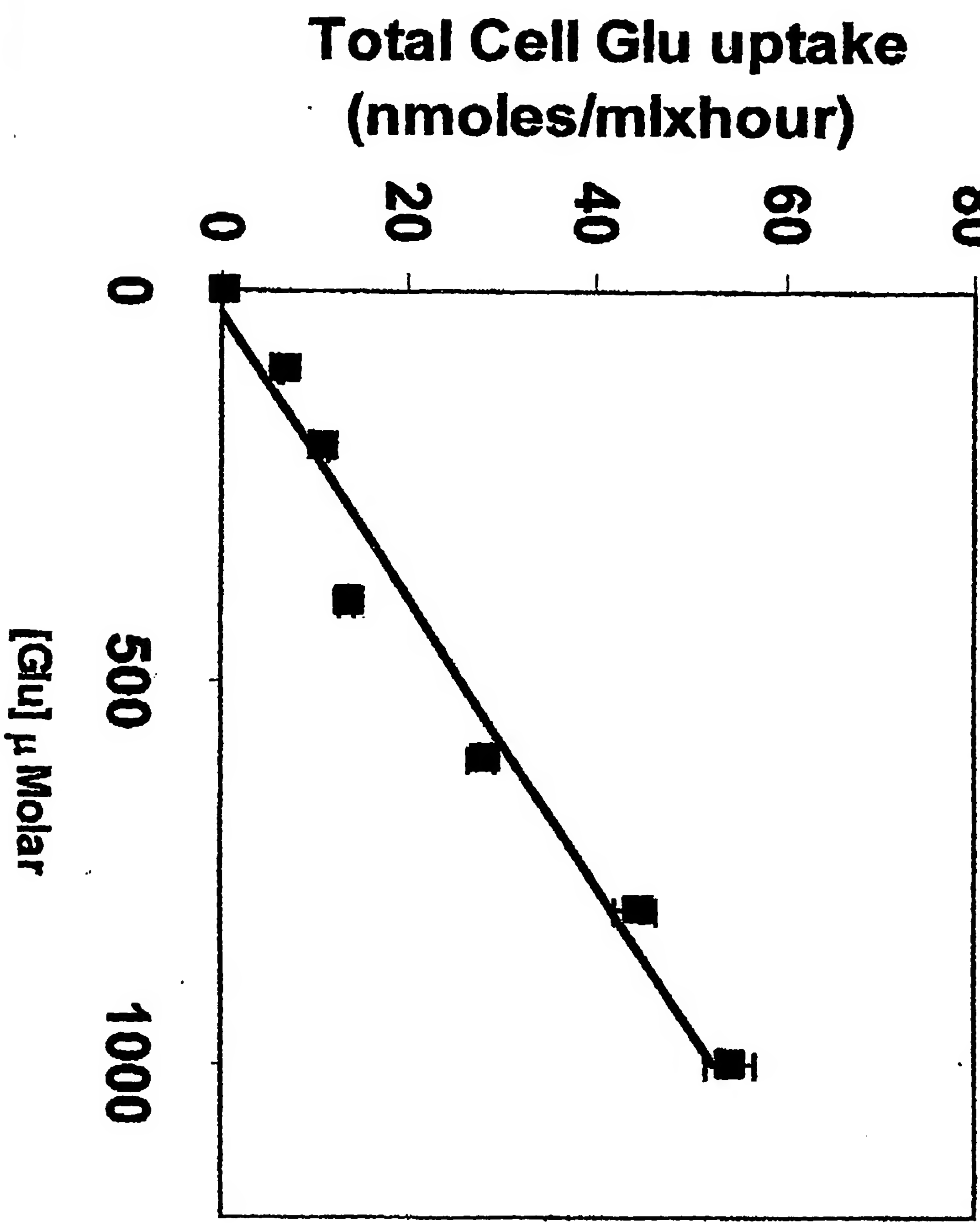

Glu (nmol/ml cell suspension)
0
20
40
60
80
100
120
140
Time (min)
0
20
40
60
80
100
120
140
160

Fig 1

50a


"Instantaneous" Glu uptake
(nmoles/ml cell suspension)
0
10
20
30
40
50
0
500
1000
[Glu]ᵀ, μM

50b


Total Cell Glu uptake
(nmoles/mlxhour)
0
20
40
60
80
0
500
1000
[Glu] μ Molar

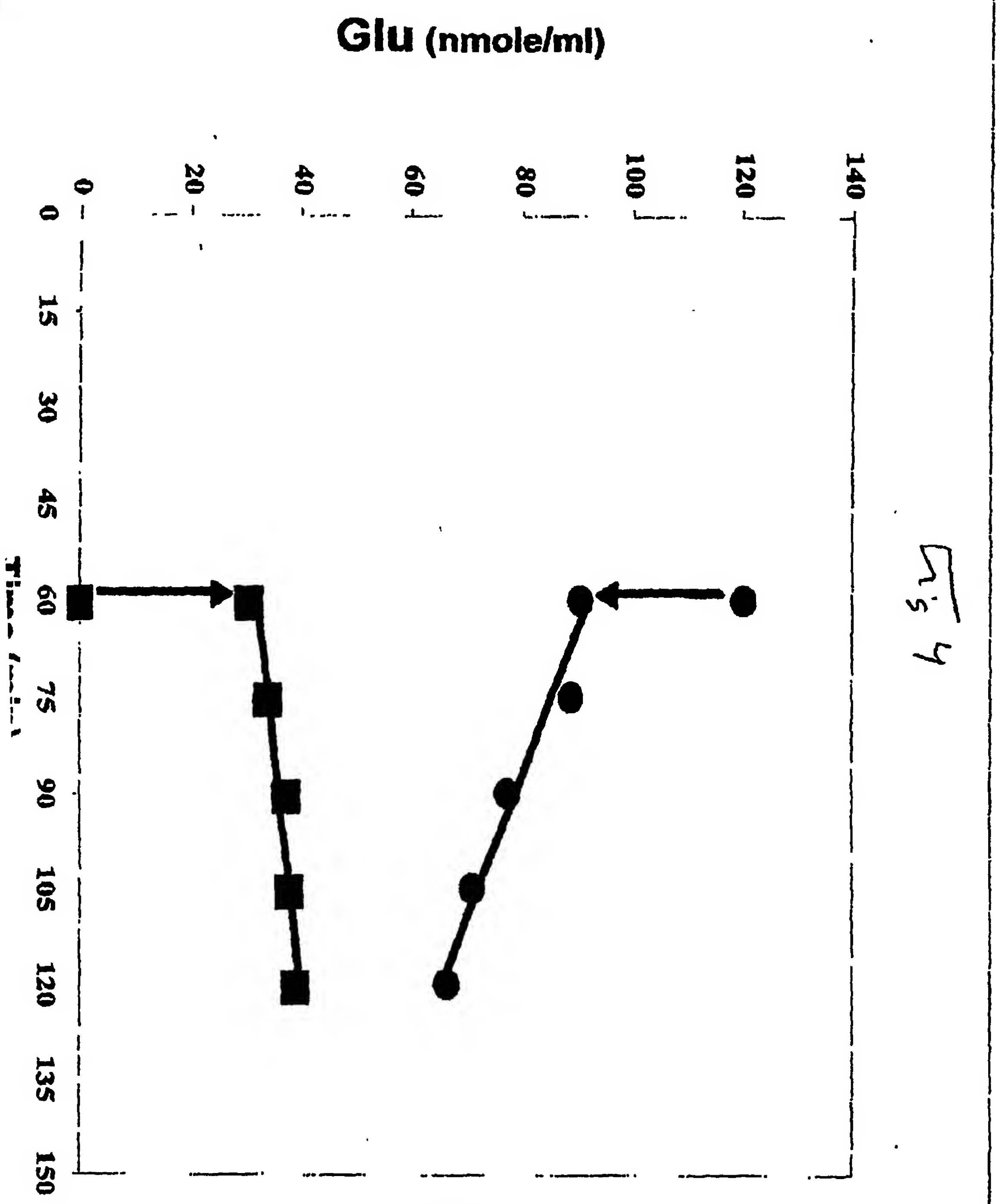
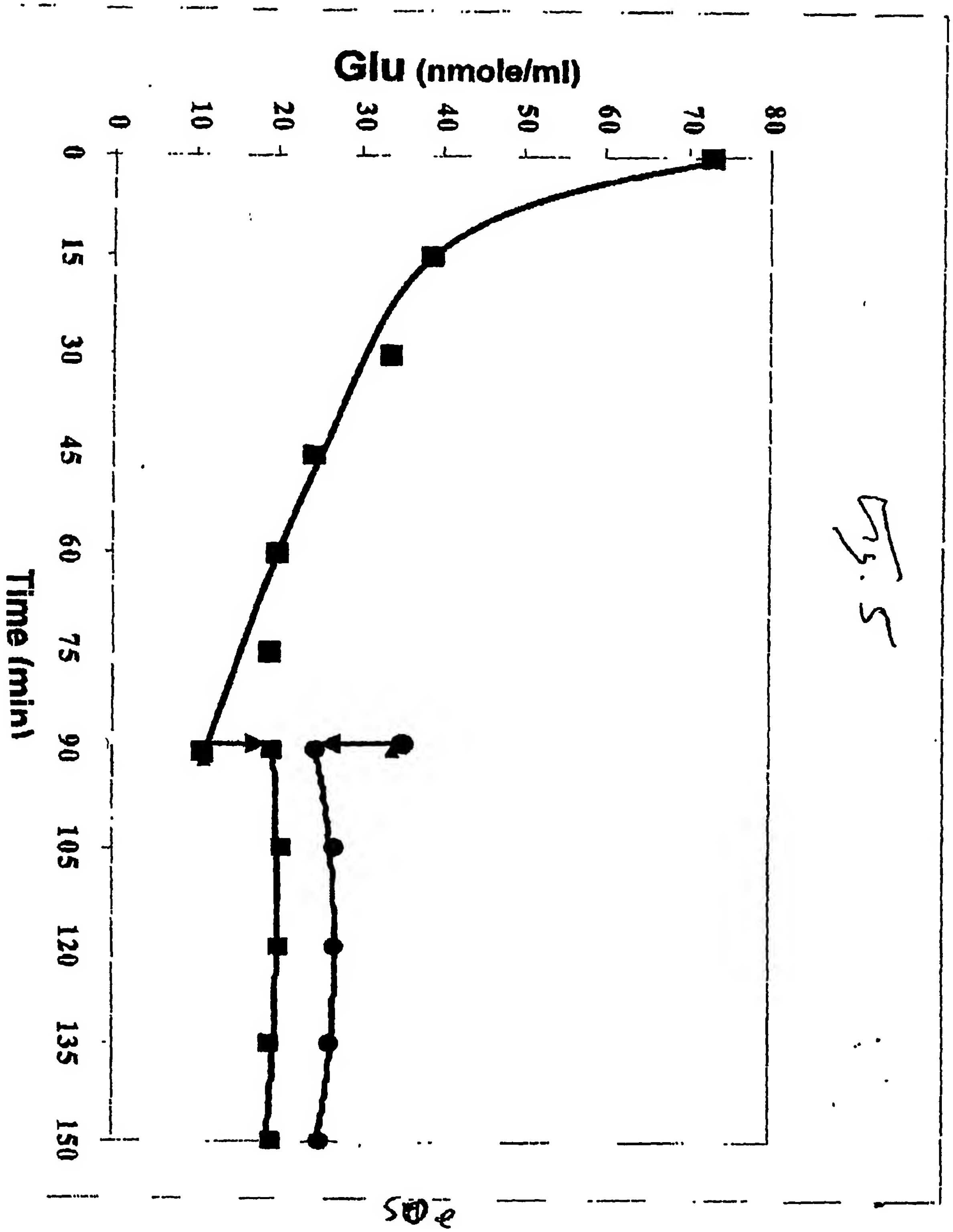


Fig 4

50d


Glu (nmole/ml)
0 10 20 30 40 50 60 70 80
Time (min)
0 15 30 45 60 75 90 105 120 135 150

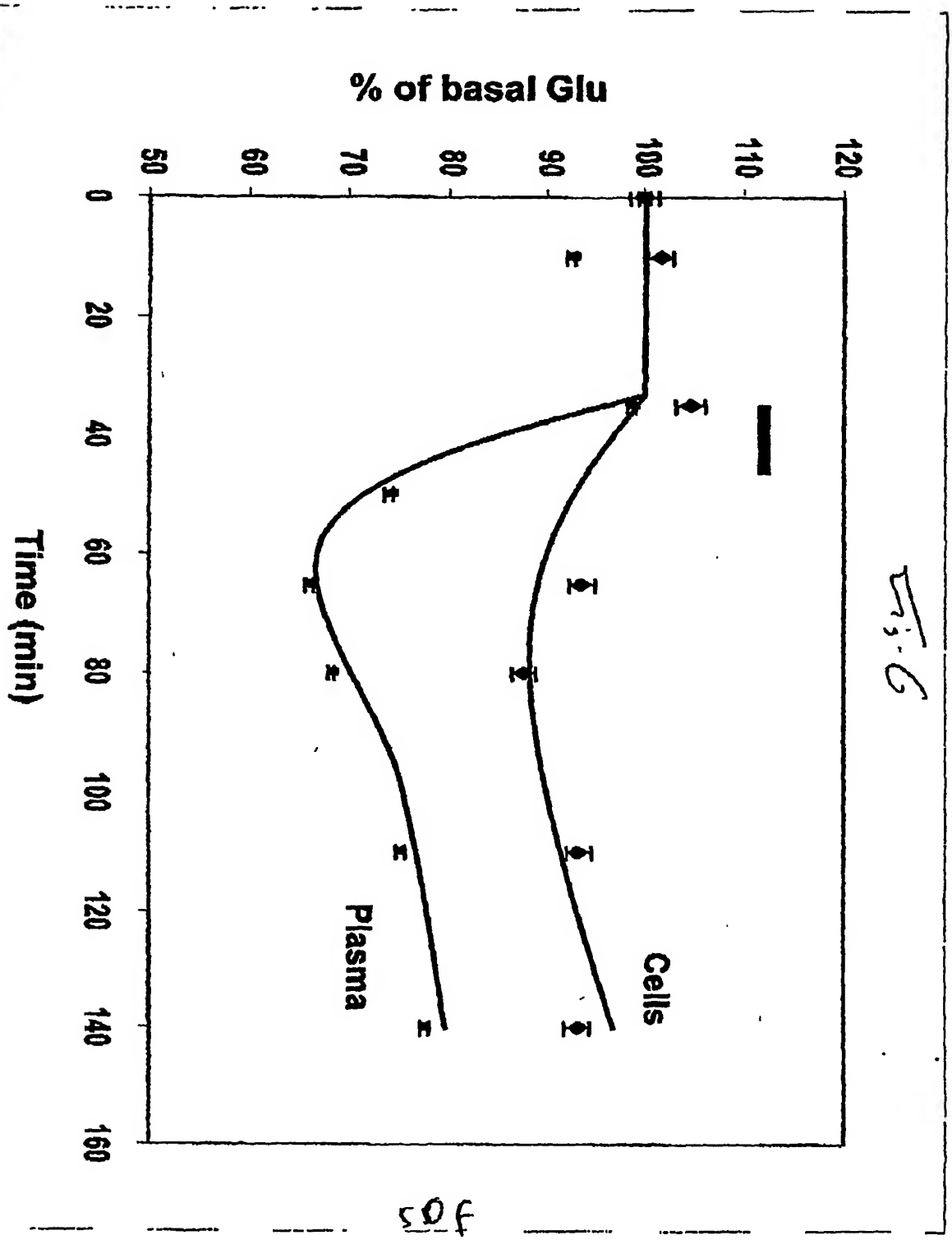


Fig. 6

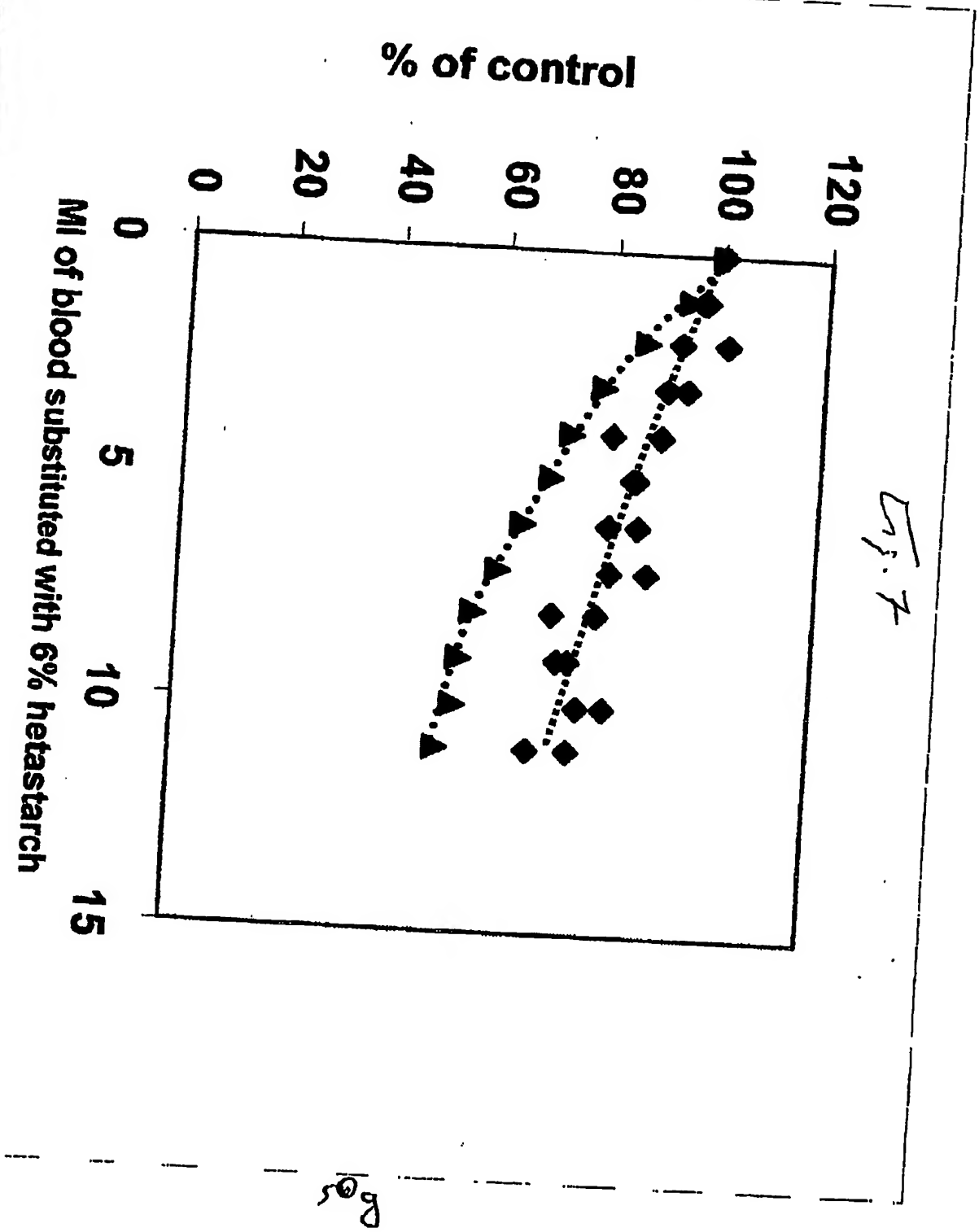

% of control
0
20
40
60
80
100
120
0
5
10
15
Ml of blood substituted with 6% hetastarch

Fig. 7

50g

D. ACCELERATED BRAIN TO BLOOD EFFLUX OF GLUTAMATE BY INTRAVENOUS PYRUVATE AND OXALOACETATE: A NOVEL APPROACH FOR THE MANAGEMENT OF NEURODEGENERATIVE DISEASES

Abstract

The feasibility of accelerating the brain to blood Glutamate efflux was studied by using two basic paradigms based on the fate of [^{3}H]Glutamate infused into brain. In the first, we infused [^{3}H]Glutamate intracerebroventricularly and followed its appearance in blood before, during and after decreasing blood Glutamate levels by the intravenous administration of the respective glutamate co-substrates of the Glutamate-pyruvate transaminase and the Glutamate-oxaloacetate transaminase, pyruvate and oxaloacetate. In the other, we performed ventriculo-cisteranl perfusions of [^{3}H]Glutamate and followed its disappearance from brain before, during and after decreasing blood Glu levels with pyruvate and oxaloacetate. In both cases, the results obtained point out to the same conclusion that the intravenous administration of pyruvate and oxaloacetate that reduces blood Glu levels also accelerates proportionally the brain to blood Glu efflux. We propose the use of pyruvate and oxaloacetate for the treatment of all neurodegenerative conditions in which the excess Glutamate present in the brain interstitial fluid or cerebrospinal fluid is thought to trigger neuronal cell death and its accompanying neuropathological sequelae.

Introduction

Abnormally high Glutamate (Glu) levels in brain interstitial and cerebrospinal fluids are the hallmark of several neurodegenerative conditions. These include acute brain anoxia/ischemia i.e stroke (Graham et al., 1993; Castillo et al., 1996), perinatal brain damage (Hagberg et al., 1993; Johnston, 1997), traumatic brain injury (Baker et al., 1993, Zauner et al., 1996), bacterial meningitis (Spranger et al, 1996), subarachnoid hemorrhage, open heart and aneurysm surgery (Persson et al., 1996; Saveland et al., 1996), hemorrhagic shock (mongan et al. 1999, 2001), newly diagnosed epilepsy (Kalviainen et al., 1993), acute liver failure (Rose et al. 2000) and various chronic neurodegenerative diseases such as glaucoma (Dreyer et al., 1996), amyotrophic lateral sclerosis (Rothstein et al., 1990; Shaw et al., 1995), HIV dementia (Ferrarese et al. 2001) and Alzheimer's disease (Pomara et al., 1992).

In animal models of some of the above disorders, it was established that glutamate receptors antagonists are able to prevent or limit the neuronal degenerative process suggesting that the latter is causally related to the Glu excitotoxicity i.e to the excessive activation of glutamate receptors by the abnormally high Glu levels and which eventually leads to neuronal death. Accordingly, clinical trials were initiated to test the efficacy of glutamate receptors antagonists particularly in the management of stroke. However, these trials had to be brought to a halt because of unacceptable side effects of the tested drugs.

In light of these failures and the need of alternative approaches to the treatment of neurodegenerative disorders involving Glu excitotoxicity, we made the hypothesis that excess Glu in brain interstitial (ISF) and cerebrospinal (CSF) fluids could possibly be eliminated by increasing a relatively poorly studied brain to blood Glu efflux. We further surmised that such increased efflux could result from lowering the Glu levels in plasma increasing thereby the driving force for Glu fluxes from brain ISF/CSF to blood.

In the accompanying papers, we established the in vitro (Wang et al. 2002) and in vivo (Gottlieb et al. 2002) conditions allowing a decrease of plasma Glu by

activation of two blood resident enzymes Glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) by administration into blood of the Glu co-substrates pyruvate and oxaloacetate.

In the present paper, we provide evidence that the intravenous administration of pyruvate and oxaloacetate causes an increased Glu efflux from brain ISF/CSF to blood.

Materials and Methods

Glutamate, sodium pyruvate, sodium oxaloacetate, NADH, lactate dehydrogenase and malate dehydrogenase were from Sigma. Glutamate dehydrogenase was from Boeringer. [^{3}H]Glu (42 Ci/mmole) was purchased from Amersham.

Intracerebroventricular injections.

Sprague-Dawley rats (250-300g) were anaesthesized with an intraperitoneal injection of urethane 0.125g/0.2 ml for 100g body weight. Catheterization of the tail vein (for drug injections) and of the femoral vein (for blood aliquots withdrawals) were performed using PE10 polyethylene tubings linked to PE50 polyethylene tubing. All catheters were secured with 5-0 silk thread and flushed with heparin (3-5 microliter of 182 U/ml). A steal cannula made out from a 27G needle was implanted in the right lateral ventricle using the following stercotactic coordinates: 0.8 mm posterior to bregma; lateral 1.4 mm; depth: 4 mm from skull or 3.5 mm from dura. [^{3}H]Glu solutions in phosphate buffered saline (PBS) were injected into the lateral ventricle through the implanted cannula using a Hamilton syringe (25µl) connected to PE20 tube filled with solution. A total volume of 11µl was injected free-hand in approximately 2min. For radioactivity determination- 50 microliter blood samples were diluted in 500µl H_2O and added to 16ml of scintillator. Measured cpms were corrected for quenching as determined by comparing the measured cpms of a set volume of [^{3}H]Glu added to water or to diluted blood.

Body temperature was maintained with a lamp and rectal temperature was monitored. Rat pulse rate was monitored using a Periflux system 500 and a laser Doppler probe placed onto the skull.

Intravenous injections of pyruvate and oxaloacetate diluted in phosphate buffered saline (PBS) were carried out at a rate of 0.05 ml/minute for 30 min with a Pharmacia pump P-1. During injections and at several time points after the injections (in general, every 15 min), aliquots of 150 microliter blood were removed from the femoral vein.

Ventriculo-cisternal perfusion

Ventriculo-cisternal perfusion was carried out according to the procedure described by Davson et al ( ). 27 G cannulas were placed in the two lateral ventricles and another into the cisterna magna. The two cannulas implanted in the lateral ventricles were connected to PE10 polyethylene tubings attached to two 5 ml syringes. The latter syringes were driven by a Harvard apparatus infusion pump to release 26 microliter/min of [^{3}H] Glu in artificial CSF (122mM NaCl, 25 mM $NaHCO_3$; 3 mM KCl; 1.4 mM $CaCl_2$; 1.2 mM $MgCl_2$ 0.4mM K_2HPO_4; 10 mM HEPES, 10 mM glucose, pH 7.42). Each syringe contained 4 ml of artificial CSF, 0.2 micromolar [^{3}H]Glu and, when needed , various amounts of non-radioactive Glu . The cannula implanted in the cisterna magna was connected to a PE10 tubing with its outlet kept 17.5 cm belong the aural line. Upon infusion into the lateral ventricles, the fluid emerging from the cisterna was collected as a function of time.

Glu determination

Whole blood and plasma samples were deproteinized by adding an equal volume of ice-cold 1M perchloric acid (PCA) and then centrifuging at 16000×g for 10 min at 4^{0}C. The pellet was discarded and supernatant collected, adjusted to pH 7.2 with 2M K_2CO_3 and, if needed, stored at -20^{0}C for later analysis.

Glutamate concentration was measured in the supernatant using the fluorometric method of Graham and Aprison (1966) A 20microliter aliquot from PCA supernatant was added to 480microliter HG buffer containing 15 U of

glutamate dehydrogenase in 0.2mM NAD, 0.3M glycine, 0.25M hydrazine hydrate adjusted to pH 8.6 with 1N H_2SO_4 . After incubation for 30-45 min at room temperature, the fluorescence was measured at 460 nm after excitation at 350 nm. A glutamate standard curve was established with concentrations ranging from 0-6 micromolar. All determinations were done at least in duplicates. The results are expressed as mean.+-SD. When CSF Glu levels were measured, the enzymatic cycling method of Kato et al. (1973) was applied.

Results

To monitor the effects of the reduction of plasma Glu on the fate of brain Glu, three different experimental systems were investigated. In the first, radiolabelled Glu was injected into a lateral ventricle and the appearance of radioactivity in blood was followed up with time, while plasma Glu levels were either not affected or decreased by the intravenous administration of pyruvate and oxaloacetate. In the second, a ventriculo-cisternal perfusion of radiolabelled Glu was performed and the percentage of Glu absorbed was measured, while plasma Glu levels were either not affected or reduced by the intravenous administration of pyruvate and oxaloacetate. In the third, the evolution of the CSF Glu levels was monitored during and following the reduction of plasma Glu by the intravenous administration of pyruvate and oxaloacetate.

Figure 1 shows the evolution of radioactivity in blood following the intracerebroventricular injection of 10 microCi of [^{3}H]Glu. It can be seen that it takes place in two phases: during the first phase, radioactivity appears in blood as soon as [^{3}H]Glu is injected in the lateral ventricle and increases linearly up to 10 min; in the second phase, blood radioactivity remains constant for at least 40 min. During the first phase, the initial rate of appearance of radioactivity in blood is 0.8% ± 0.1(n=8) of the radioactivity input in brain/per min which corresponds to a half-life of elimination of [^{3}H]Glu from brain of 62.5 ± 7 min. The intuitive explanation for the

second phase is that it corresponds to a steady state between the brain to blood [^{3}H]Glu efflux rate and the rate of disappearance of [^{3}H]Glu from blood.

To estimate the rate of disappearance of [^{3}H]Glu from blood i.e the Glu life time in blood, we carried out bolus intravenous injections of 1 microCi radiolabeled Glu in the absence or presence of non radioactive Glu and monitored the radioactivity in blood with time. Figure 2 shows that, as expected, the evolution of [^{3}H]Glu radioactivity in blood takes place in two phases: a fast phase terminating within less than 60 seconds and which corresponds most likely to the distribution phase and a slow one that corresponds to the elimination phase. Accordingly, one can calculate the elimination half-life of [^{3}H]Glu in blood as being 19.3 min (and an elimination rate constant equal to ln2/ 19.3 = 0.035 min^{-1}) for a normal blood Glu concentration of 209 micromolar 30 (SD; n=34), and thus that 5.4 nmoles of Glu/ml blood are eliminated per min. The elimination half-life increases to 24.6 min when blood Glu is made to reach a steady state concentration of 3.4mM by intraperitoneal administration of 2 mMoles of Glu. Under these conditions, 69 nmoles Glu/ml blood are eliminated per min. Thus, the rate of Glu elimination increases by about 13 fold for a 16 fold increase of blood Glu concentration suggesting that the enzymes and transporters involved in Glu elimination are not yet saturated at 3.4 mM. The fact that nonradioactive Glu slows down the elimination half life of [^{3}H]Glu is compatible with the suggestion that blood radioactivity corresponds mainly to authentic [^{3}H]Glu and that Glu and [^{3}H]Glu compete for the same transport sites in organs that display an avid Glu uptake from blood such as muscle, kidney, intestine and lung (Hediger and Welbourne, 1999). In line with the above, Hoyosa et al (1999) have indeed observed that L-Glu in the brain interstitial fluid is transported across the blood brain barrier in an intact form.

Figure 3 shows the evolution of radioactivity in blood following the intracerebroventricular injection of 10 microCi of [^{3}H]Glu. In this experiment, once a steady state level of radioactivity was reached in blood, the Glu blood levels were transiently decreased by the intravenous administration of pyruvate and oxaloacetate.

It can be seen that the evolution of blood radioactivity originating from brain displays an almost mirror image to that of blood Glu. While the latter decreases by about 25% during the administration of pyruvate and oxaloacetate and then increases, the blood radioactivity increases by about the same percentage and then decreases. It appears thus that the decrease of blood Glu causes an increased [^{3}H]Glu efflux from brain. The results presented in Figure 4 were further analyzed as follows.

Figure 4 shows the evolution of brain [^{3}H]Glu as a function of time calculated on the basis of the data presented in Figure 2 according to the relation I:

$$C_t = C_0 - (R_t - R_{(t-Dt)} \times e^{-K(t-Dt)}) \qquad (I)$$

where C_t = amount of [^{3}H]Glu remaining in brain at time t; C_0 = amount of [^{3}H]Glu in brain at time t = 0; R_t = amount of [^{3}H]Glu in blood at time t; $R_{(t-Dt)}$ = amount of [^{3}H]Glu remaining in blood from time t-Dt; K = elimination rate constant of [^{3}H]Glu in blood (=ln2/ elimination half life of [^{3}H]Glu in blood) ; thus, $(R_t - R_{(t-Dt)} \times e^{-K(t-Dt)})$ = net release of [^{3}H]Glu from brain during the time interval of Dt. It can be seen from Figure 4 that after the first 10 min brain [^{3}H]Glu decreases in three phases. Before and after the administration of pyruvate and oxaloacetate, the residence half-lifes of [^{3}H]Glu in brain is 201 min and 199 min respectively while it decreases to 153 min during the administration of the GPT/GOT substrates. Thus, the latter substrates decrease the residence time of brain [^{3}H]Glu in line with the concept of an accelerated brain to blood Glu efflux.

An additional way to analyze the data presented in Figure 4 is to calculate the fractional rate of [^{3}H]Glu release F given by the relation II:

$$F = (R_t - R_{(t-Dt)} \times e^{-K(t-Dt)}) / C_{(t-Dt)} \qquad (II)$$

where $C_{(t-Dt)}$ = amount of [^{3}H]Glu remaining in brain at time t-Dt

Figure 5 shows the F values calculated before, during and after the administration of pyruvate and oxaloacetate. Again, it can be seen that the rate of Glu release from brain is increased by about 50% by the intravenous administration of pyruvate and oxaloacetate following which it returns back to its original value. It should be mentioned that this increase is abolished when [^{3}H]Glu is injected in a solution containing nonradioactive Glu at a 5 mM concentration indicating that the brain to blood efflux of Glu is saturable.

In order to provide further evidence that the intravenous administration of pyruvate and oxaloacetate that reduces blood Glu levels can influence the extracellular levels of brain Glu, we carried out experiments of ventriculo-cisternal perfusion of [^{3}H]Glu and measured the extent of its elimination from the perfused fluid. In this paradigm, a [^{3}H]Glu containing solution is continuously perfused through cannulas implanted in the lateral ventricles and is collected as it emerges from a cannula implanted in the cisterna magna. The ratio R of the radioactivity input per unit volume to that of the output, provides an index of the percentage of [^{3}H]Glu absorbed from the perfused fluid. Figure 6 shows that the ventriculo-cisternal perfusion with a [^{3}H]Glu solution at a concentration of 0.2 micromolar leads to a steady state absorption of about 55%. When the perfusion solution is switched to one containing 0.2 micromolar [^{3}H]Glu together with 250 micromolar of unlabeled Glu, the absorption of [^{3}H]Glu decreases to 35% because of the competition for the brain absorption sites by unlabeled Glu. These absorption sites consist mainly of the Glu transporters present on the neurons and glia and those associated with the antiluminal membranes of brain capillary endothelial cells that are responsible for the brain to blood efflux of Glu. Though their respective contributions to the extent of Glu absorption from the perfusion fluid is not known, one expect the latter absorption to increase if during the ventriculo-cisternal perfusion, the brain-to-blood efflux of Glu is accelerated. Figure 7 illustrates the fact that the intravenous administration of pyruvate and oxaloacetate increases the percentage of [^{3}H]Glu removed from the perfusion fluid from 30% to 40%.

Figure 8 illustrates the finding that, as expected, the intravenous administration of the GOT/GPT co-substrates causes a reduction of ISF/CSF Glu. In these experiments, CSF aliquots were collected from a cannula implanted in the cisterna magna, before, during and after the the intravenous administration of pyruvate and oxaloacetate. The CSF Glu levels were determined here by the cycling method ogf Kato et al. (1973). It can be seen that under conditions that cause a 50% decrease of blood Glu levels (Gottlieb et al. 2002), a parallel decrease is observed in Glu CSF.

Discussion

In this paper, we examined the prediction that a decrease of blood Glu levels should increase the ability of brain to remove Glu from the ISF/CSF into blood. Though known for more than 40 years (Berl et al. 1961), the latter process of brain to blood Glu efflux has attracted so far little attention. However, the very extensive vascularization of the brain parenchyma with an average intercapillary distance of only 24 micromolar (Pawlik et al. 1981) and the presence of Glu transporters on the antiluminal (brain) side of the blood capillaries (O'Kane et al. 1999) strongly suggest that the homeostasis of Glu in the brain extracellular spaces is maintained not only by the glial and neuronal Glu transporters but also by those present on the brain capillary endothelial cells that are responsible for the removal of Glu from brain to blood. Although the relative contributions of the various Glu transporters to the removal of extracellular Glu have not been quantified yet, it is clear that the brain capillary Glu transporters play an important role in keeping the brain interstitial fluid Glu concentration below excitotoxic levels. Indeed, it has been observed that 40% of [^{3}H]Glu microinjected in the rat parietal cortex was eliminated from brain in 20 min (Hosoya et al. 1999). This suggests that for the individual Glu molecule present in the extracellular space that has the option to either remain in brain by interacting with glial and neuronal Glu transporters and with various Glu receptor binding sites, or be removed to blood by interacting with the Glu transporters on capillary endothelial

cells, the probability of interacting with the latter is larger than with any of its other molecular targets.

The efflux rate of 2%/min measured by Hosoya et al. (1999) is faster by a factor of 3 than the initial rate of Glu efflux observed here. This difference can possibly be accounted by differences in the Glu concentration around the transporters since the dilution in CSF of [^{3}H]Glu injected intracerebroventricularly is likely to be larger than that taking place in the restricted space of the cortical interstitial fluid.

To interpret our results, we tend to regard the CSF as a reservoir of diluted [^{3}H]Glu that keeps feeding, at least for some time, the brain parenchyma. Once it has diffused from the CSF into the ISF, [^{3}H]Glu is removed at a steady rate into blood. Such a scheme can account for the observation made in Figure 1 that the intracerebroventricular injection of [^{3}H]Glu, seen from the blood perspective, is characterized by an initial fast increase followed by a steady state during which the blood [^{3}H]Glu levels remain constant in spite of the short [^{3}H]Glu life time in blood. Accordingly, one may surmise that this steady state corresponds to an equilibrium between the rate of [^{3}H]Glu efflux from brain and the rate of Glu elimination from blood. As we estimated the latter from Figure 2 to have a rate constant of 0.035 min^{-1}, it follows that the brain to blood [^{3}H]Glu efflux rate constant is identical to the value of 0.0346 min^{-1} reported by Hosoya et al. (1999).

We have attempted to demonstrate here the feasibility of accelerating the brain to blood Glu efflux by using two basic paradigms based on the fate of radiolabeled Glu infused into brain. In the first, we followed its appearance in blood before, during and after decreasing blood Glu levels. In the other, we followed its disappearance from brain before, during and after decreasing blood Glu levels. In both cases, the results obtained point out to the same conclusion that the intravenous administration of pyruvate and oxaloacetate that reduces blood Glu levels also accelerates the brain to blood Glu efflux. This acceleration manifests itself, in the experiments of intracerebroventricular infusion of [^{3}H]Glu, by the fact that the intravenous administration of pyruvate and oxaloacetate, which decreases blood Glu

levels by about 25%, causes both a parallel increase of blood radioactivity originating from brain, a decrease of the [^{3}H]Glu residence time in brain and an increase of the fractional rate of [^{3}H]Glu efflux from brain. In the experiments of ventriculo-cisternal perfusion of [^{3}H]Glu, it manifests itself by the fact that the intravenous administration of pyruvate and oxaloacetate causes a 33% increased [^{3}H]Glu removal from the perfused solution. The intravenous administration of pyruvate and oxaloacetate also decreases the basal CSF Glu levels. That a decrease of blood Glu causes an increased Glu efflux from brain confirms the early observation of Drewes et al. (1977) that perfusion of the dog brain with plasma containing lower Glu levels than normal caused a larger efflux of brain Glu.

The results obtained also reveal that the amplitude and duration of the decrease of CSF/ISF Glu levels match those of the blood Glu levels, thus suggesting that the latter control in fact the movements of Glu from brain to blood. Though upon discontinuation of the pyruvate and oxaloacetate administration, the Glu levels tend to recover their original values both in blood and in the CSF/ISF, the two recovery processes are unlikely to be linked because of the relative impermeability of the blood brain barrier to a Glu influx from blood into brain. While the recovery in blood can be mainly attributed to a reparative increased Glu efflux from Glu-containing organs such as liver and muscle (Rediger and Welbourne, 1999), the recovery of the CSF/ISF Glu levels is probably due to an enhanced Glu leakage from neurons and glia. The kinetics of recovery of ISF/CSF Glu levels is in fact a critical factor in the proposed strategy of decreasing blood Glu levels for therapeutic purposes. In the various cases in which the abnormally high CSF/ISF Glu levels are suggested to have an important pathological role, the kinetics of recovery of Glu following its blood-mediated depletion will dictate whether the treatment with pyruvate/oxaloacetate will be effective or not. Thus, in some cases, a transient decrease of CSF/ISF Glu levels may suffice while, in others, a repetitive or sustained decrease would be required. The latter objective could possibly be achieved by the use of pyruvate/oxaloacetate

prodrugs or of slow release formulations. In emergency cases, a prolonged intravenous feeding might be warranted.

What is the mechanism by which pyruvate and oxaloacetate cause an enhanced brain to blood Glu efflux? Figure 9 presents a plausible mechanism based on the model of Lee et al. (1998) and O'Kane et al. (1999). Na^+-dependent Glu and glutamine transporters are present at the antiluminal membrane (brain facing) of brain capillary endothelial cells while facilitative systems for these amino acids are located on the luminal membrane (blood facing). Driven by the large Na^+ gradient, the transporters take up and concentrate Glu and glutamine from the brain ISF into the endothelial cells. A phosphate-dependent glutaminase present within the endothelial cells hydrolyzes glutamine into Glu and ammonia helping thereby to create an intracellular Glu concentration larger than that of plasma. The facilitative glutamate carriers at the luminal membrane allow then Glu to move from the endothelial cells into blood plasma down its electrochemical gradient. There is however an additional process whereby the Glu transporters, saturated with intracellular Glu, are activated by the intracellular Na^+ ions and allow some Glu together with Na^+ ions to move back in the ISF. This "reverse pumping" process together with the action of the Na^+/K^+ ATPase ions prevents the accumulation of intracellular Na^+ ions. The reduction of blood of Glu levels increases the Glu gradient and accelerates the efflux of Glu from the endothelial cells into plasma. As the Glu transporters are no more saturated with Glu, the "reverse pumping" process is greatly diminished and therefore the net Glu flux from the ISF into the endothelial cell is increased.

The present study and the accompanying papers (Wang et al. 2002; Gottlieb et al. 2002) emphasize the ability of pyruvate/oxaloacetate to decrease both blood and CSF/ISF Glu levels and suggest the possible use of these natural GPT and GPT co-substrates for therapeutic purposes in the context of the large number of neurodegenerative conditions in which the excess ISF/CSF Glu is thought to exert a crucial excitotoxicity. Though the neuroprotective properties of oxaloacetate have

not been studied so far, those of pyruvate are well established but have not been used yet in the clinic. In the last fifteen years, several publications have emphasized the in vitro protective effects of pyruvate on cultured cells in general (Andrae et al. 1985) and neurons in particular (Izumi et al. 1994; Matsumoto et al. 1994; Desagher et al. 1997; Ruiz et al. 1998; Hodgkins et al. 1999;Maus et al. 1999). Pyruvate was also found to display neuroprotective properties in in vivo conditions such as hemorrhagic shock (Mongan et al. 1999; 2001; Fink, 2002) and transient forebrain ischemia (Lee et al. 2001). In the studies of Mongan et al. (1999,2001), a 4 hour-long controlled arterial hemorrhage was performed in a swine and the effects of intravenous pyruvate were evaluated on various parameters including neocortical function and ischemia. Thirty minutes after the onset of hemorrhagic shock, pyruvate was infused to attain arterial levels of 5 mM. During hemorrhage, the pyruvate treated group was found to exhibit improved cerebral cortical metabolic and function status as assessed by the improved preservation of the electrocorticogram. Most importantly and of prime relevance to the present study, intravenously administered pyruvate was found to prevent the eightfold increase in ISF glutamate observed in the control group and resulting from hemorrhagic shock. The authors tend to account for the observed neuroprotective effects of pyruvate by suggesting it improves the neuronal energy status. They base this argument on their finding that pyruvate crosses the blood-brain barrier and thus is available for use by neuronal tissue to enhance metabolic stability during the prolonged hemorrhagic hypotension. However, the brain microdialysate pyruvate levels that were measured in the pyruvate-treated group characterized by an arterial pyruvate concentration of 5 mM were found to increase only from 0.09 to 0.43 mM, a concentration that is unlikely to significantly support the neuronal energy status. We therefore suggest to reinterpret the results of Mongan et al. (1999,2001) on the basis of a blood pyruvate-mediated enhanced brain to blood Glu efflux. It is interesting to mention that Glu accumulation in the brain microdialysate, which is significant from 60 min up to 150 min after the start of hemorrhage, takes place with an approximate rate of 0.4%/min which is slightly less than the 0.5%/min fractional

brain efflux rate produced by pyruvate and oxaloacetate. In our experiments, both substrates were present in blood at the concentrations of only 0.45 mM (Gottlieb et al. 2002) but their effects are synergistic (Wang et al. 2002).

In the study of Lee et al (2001), rats injected intraperitoneally with sodium pyruvate (500-1000 mg/kg) within 1 hr after 12 min forebrain ischemia showed almost no neuronal death neither in the hippocampus nor in the cortex. In addition, the mortality was markedly decreased in the pyruvate-protected groups (3.8%) compared with the NaCl-injected control group (58.1%) and the neuroprotective effect persisted even at 30 days after the insult. Since forebrain ischemia causes an increase of ISF Glu which produces the excitotoxic damage (Choi and Rothman, 1990), we suggest that here again the neuroprotective effects of pyruvate can be attributed to the fact that it decreases the blood Glu levels and accelerates thereby a beneficial brain to blood Glu efflux.

In summary, we have brought here the experimental evidence establishing that the intravenous administration of the GPT and GPT glutamate co-substrates pyruvate and oxaloacetate causes a decrease of ISF/CSF Glu levels by virtue of their ability to decrease blood plasma Glu. The enhanced brain to blood Glu efflux is suggested to account for the neuroprotective effects of pyruvate in forebrain ischemia and hemorrhagic shock. We propose the use of pyruvate and oxaloacetate for the treatment of all neurodegenerative conditions in which excess ISF/CSF Glu is thought to trigger neuronal cell death and its accompanying neuropathological sequelae.

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Legends of figures

Figure 1

Evolution of blood radioactivity following the intracerebroventricular infusion of 11 microCi in 11 microliter of [^{3}H]Glu. One representative experiment out of 8 performed.

Figure 2

Evolution of blood radioactivity following the bolus intravenous injections of 1 microCi [^{14}C]Glu in the absence (black circles) or presence (grey triangles) of 3.4mM unlabeled Glu. One representative experiment out of 3 performed.

Figure 3

Evolution of blood radioactivity (black diamonds) and blood Glu levels (grey squares) following the intracerebroventricular infusion of 11 microCi of [^{3}H]Glu and its modulation by the intravenous administration of pyruvate and oxaloacetate. The injection of the latter compounds was started at 60 min at a rate of 50 micromolarolcs of each compound/min for a duration of 30 min (black bar). This regimen causes the build up of an intravenous concentration of 0.45 mM for both pyruvate and oxaloacetate (Gottlieb et al. 2002). One representative experiment out of 9 performed.

Figure 4

Duration of [^{3}H]Glu residence in brain following intracerebroventricular infusion. The parameter on the ordinate represents C_t i.e the amount of [^{3}H]Glu

remaining in brain at time t calculated according to the relation I on the basis of the data presented in Figure 3.

Figure 5

Fractional rate of [^{3}H]Glu release F given by the relation II and calculated for the four time points (± SD) before (left column), during (middle column) and after (right column) the administration of pyruvate and oxaloacetate on the basis of the data presented in Figure 3.

Figure 6

Ventriculo-cisternal perfusion of [^{3}H]Glu and evolution of the percentage of [^{3}H]Glu absorbed. The latter was calculated as (1-R)x100 where R is the ratio of the radioactivity input per unit volume to that of the output collected at the cisterna magna. The perfusion was started with a 0.2 micromolar [^{3}H]Glu solution in artificial CSF and switched after 8 min to one containing 0.2 micromolar [^{3}H]Glu together with 250 micromolar of unlabeled Glu. The flow rate was 26 microliter/min. One representative experiment out of 3 performed.

Figure 7

Ventriculo-cisternal perfusion of [^{3}H]Glu and evolution of the percentage of [^{3}H]Glu absorbed. The latter was calculated as (1-R)x100 where R is the ratio of the radioactivity input per unit volume to that of the output collected at the cisterna magna. The perfusion of a 30 micromolar [^{3}H]Glu solution in artificial CSF was carried out at a flow rate of 26 microliter/min. After 60 min, an intravenous infusion of pyruvate and oxaloacetate was started at a rate of 50 micromolaroles /min for a duration of 50 min (black bar). One representative experiment out of 7 performed.

Figure 8

Evolution of CSF Glu upon decrease of blood Glu levels. The latter was achieved by an intravenous infusion of pyruvate and oxaloacetate at a rate of 50 micromolaroles /min for a duration of 30 min (black bar). Under these conditions an average of 50±10% reduction of blood Glu is achieved (Gottlieb et al. 2002). One representative experiment out of 5 performed.

Figure 9

Proposed mechanism for the accelerated brain to blood Glu efflux resulting from a decrease of blood Glu levels. Pyruvate and oxaloacetate administered into blood activate the blood-resident enzymes GPT and GOT respectively to decrease Glu and produce □-ketoglutarate (□-KG), alanine and aspartate. As a result, the Glu chemical gradient between the endothelial cell and plasma is increased and the intraendothelial cell Glu, built up by the Glu transporters on the antiluminal membrane and by the glutaminase action on Glutamine, moves into plasma by facilitated diffusion. The decrease of the intracndothelial Glu concentration prevents the Na^+ ion (black circles) dependent "reverse pumping" of the Glu transporters (that normally transport some Glu into brain) increasing thereby the net Glu efflux from brain to blood.

Claims:

1. A method of reducing brain glutamate levels, essentially as described and exemplified herein.

2. A method of treating a neurodegenerative disease by reducing brain glutamate levels, essentially as described and exemplified herein.

3. A method of scavenging blood glutamate by activation of resident enzymes, essentially as described and exemplified herein.

4. A method of depletion of plasma glutamate during partial or complete blood exchange, essentially as described and exemplified herein.

5. A method of accelerating brain to blood efflux of glutamate, essentially as described and exemplified herein.

6. A method of accelerating brain to blood efflux of glutamate by intravenous pyruvate and oxaloacetate, essentially as described and exemplified herein.

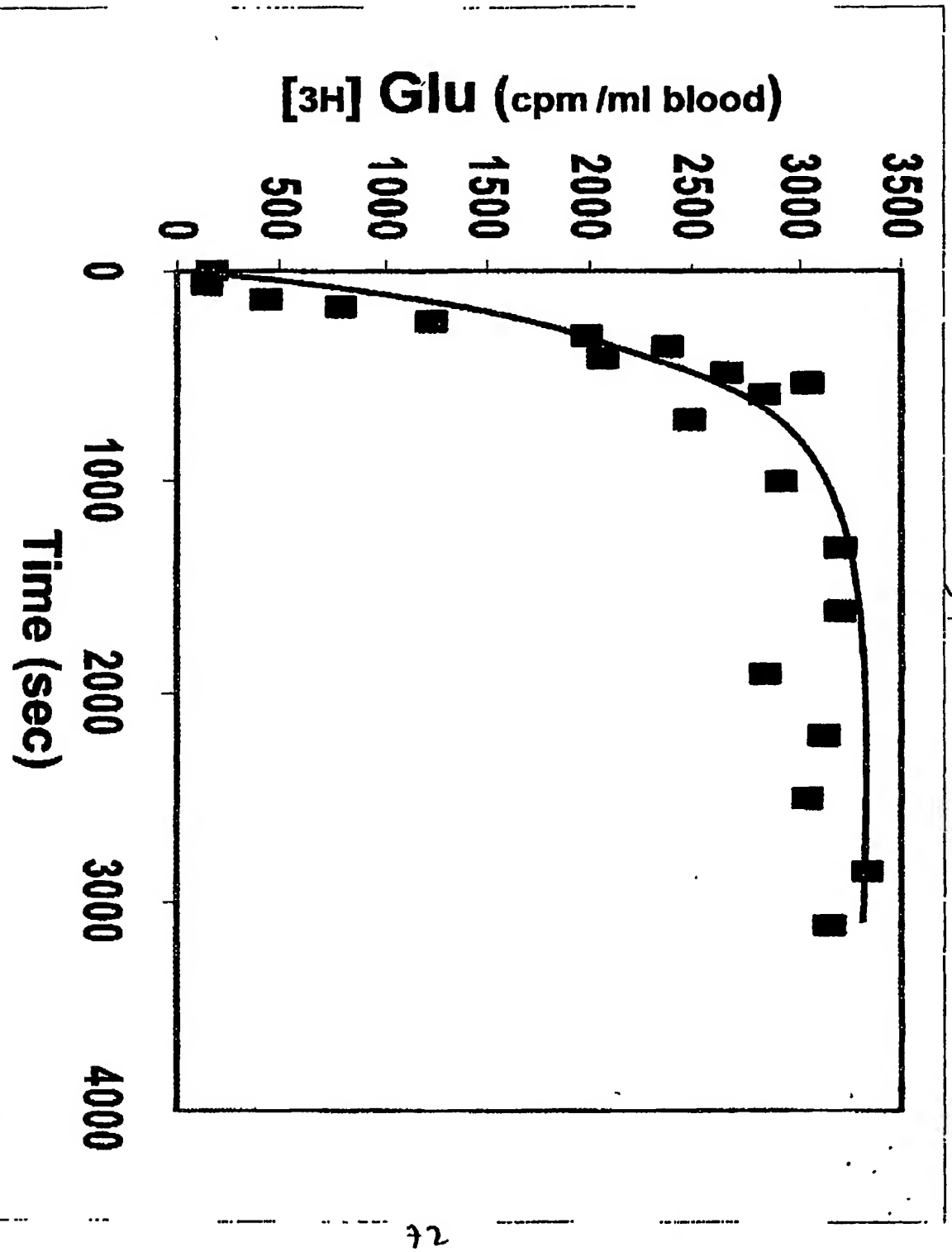


Fig. 1

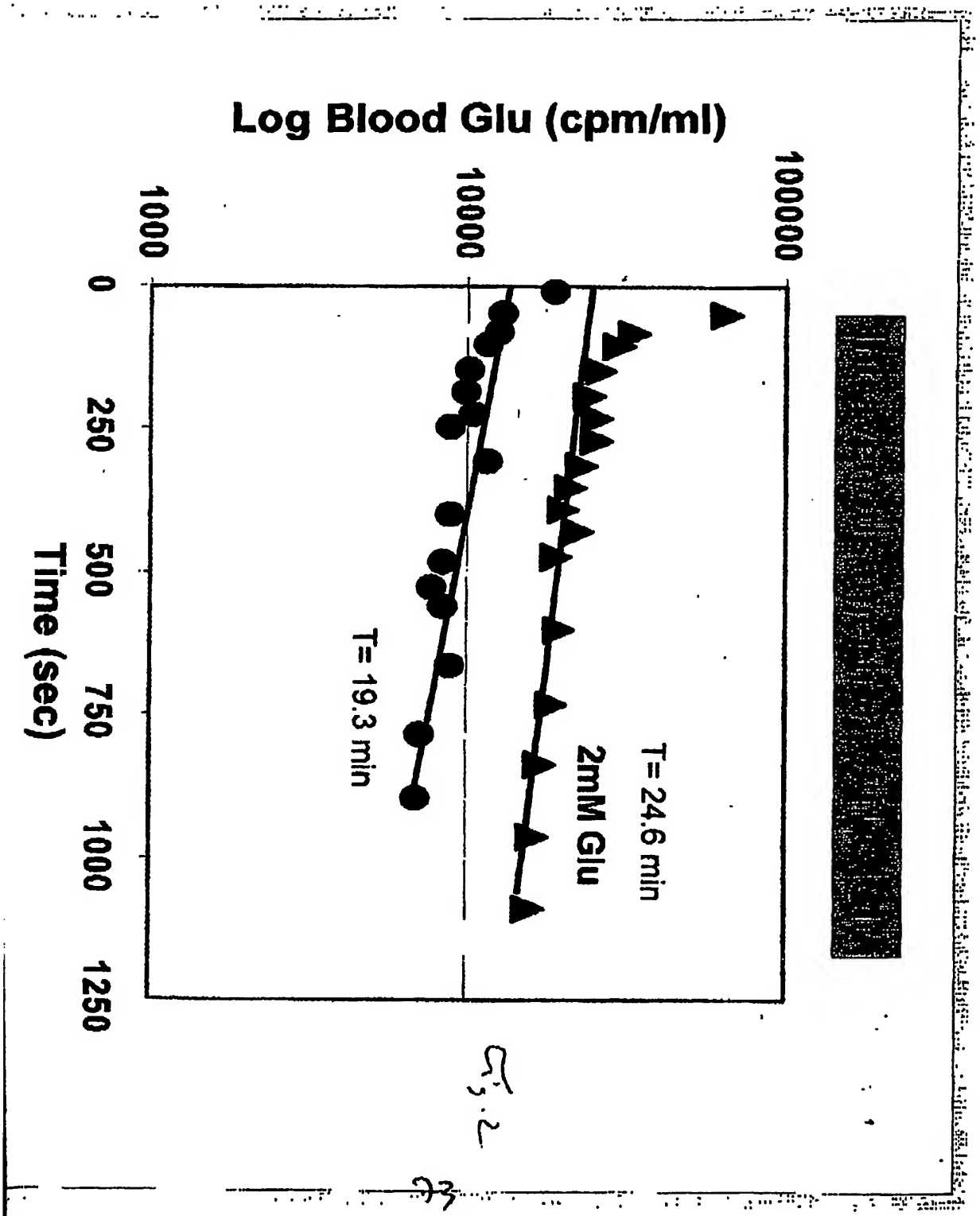


Fig. 2

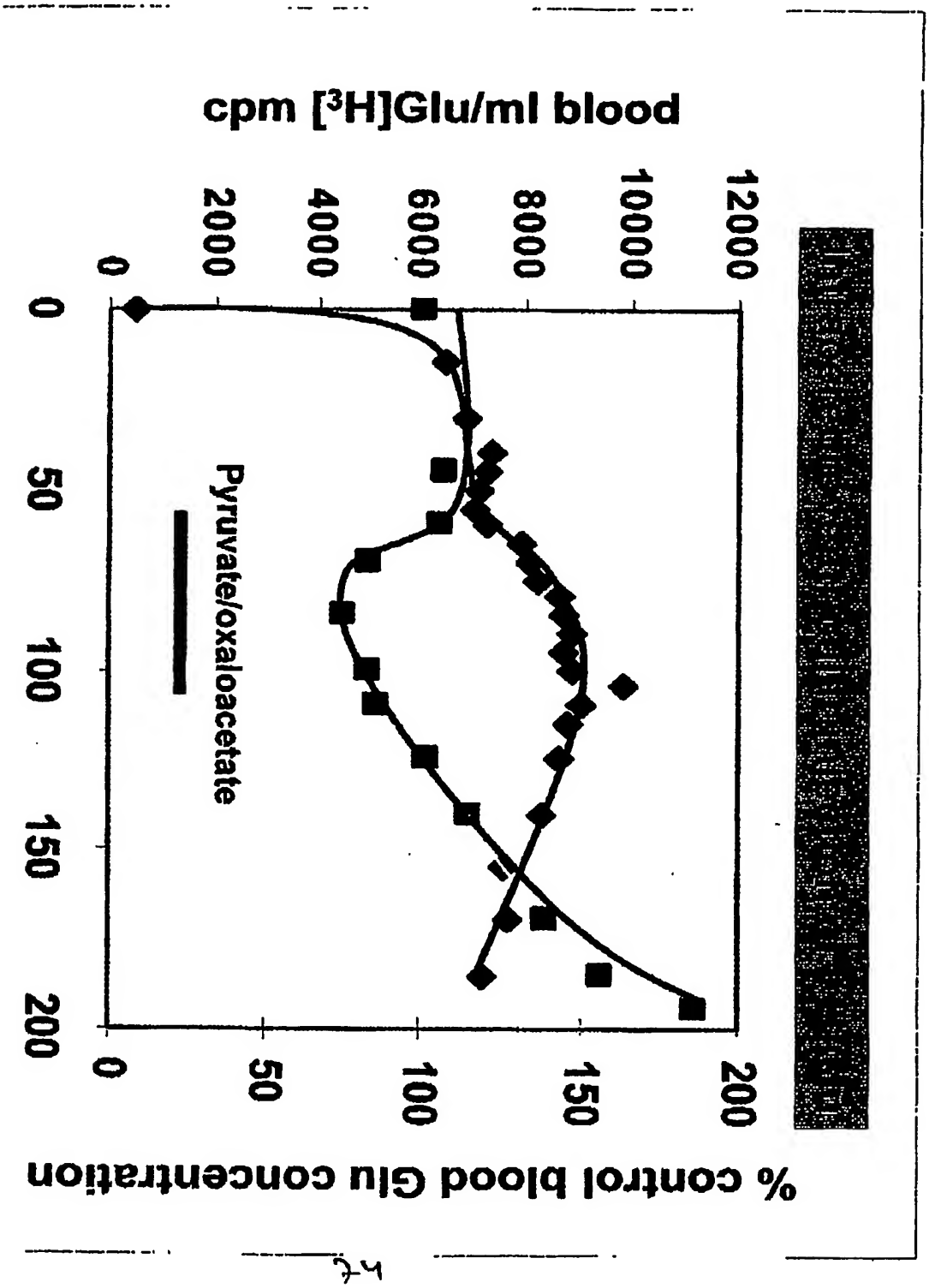

cpm [3H]Glu/ml blood
0
2000
4000
6000
8000
10000
12000
% control blood Glu concentration
0
50
100
150
200
Pyruvate/oxaloacetate

Fig 3

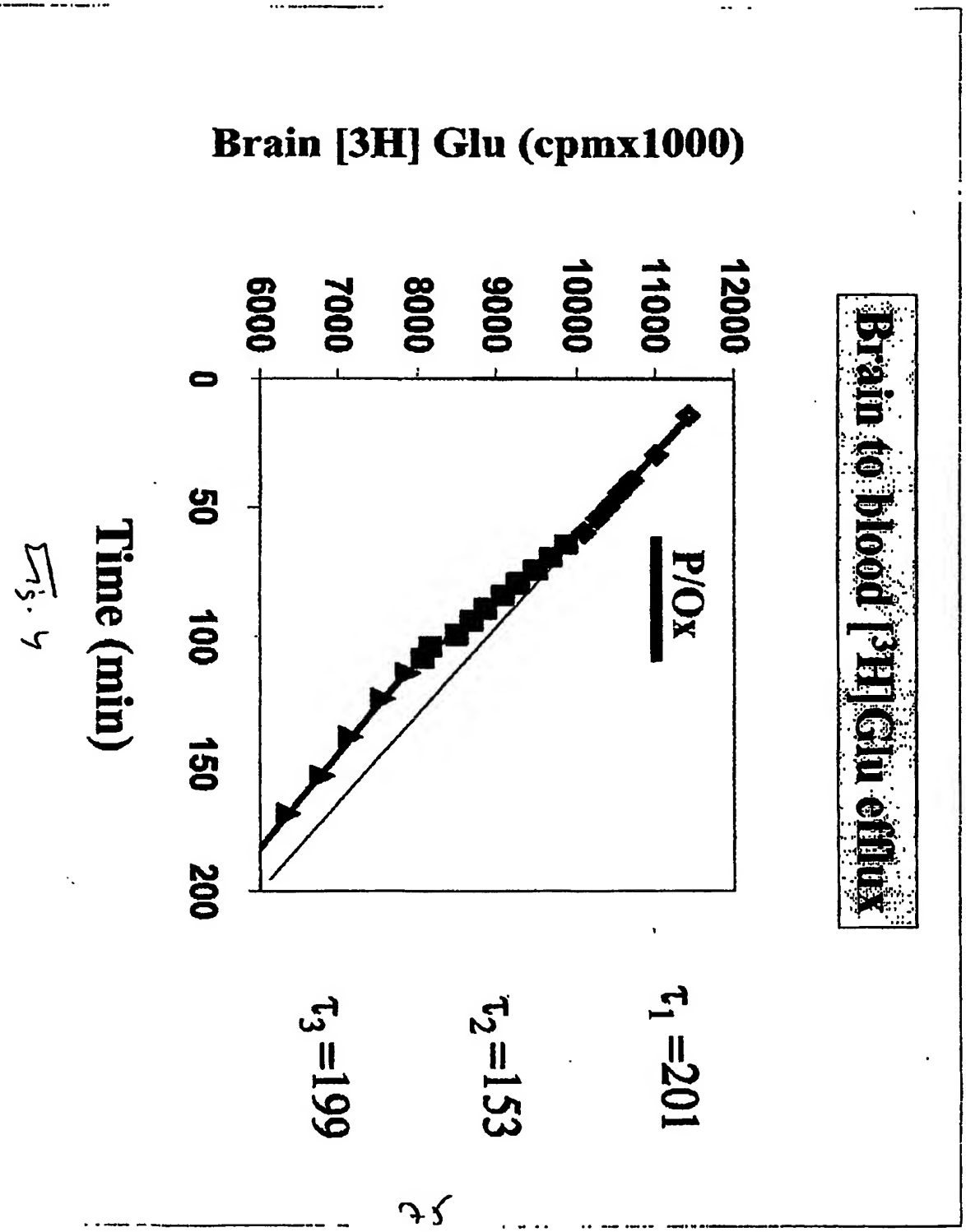

Brain [3H] Glu (cpmx1000)
Brain to blood [3H]Glu efflux
P/Ox
Time (min)
0
50
100
150
200
6000
7000
8000
9000
10000
11000
12000
τ_1 =201
τ_2=153
τ_3=199

Fig. 4

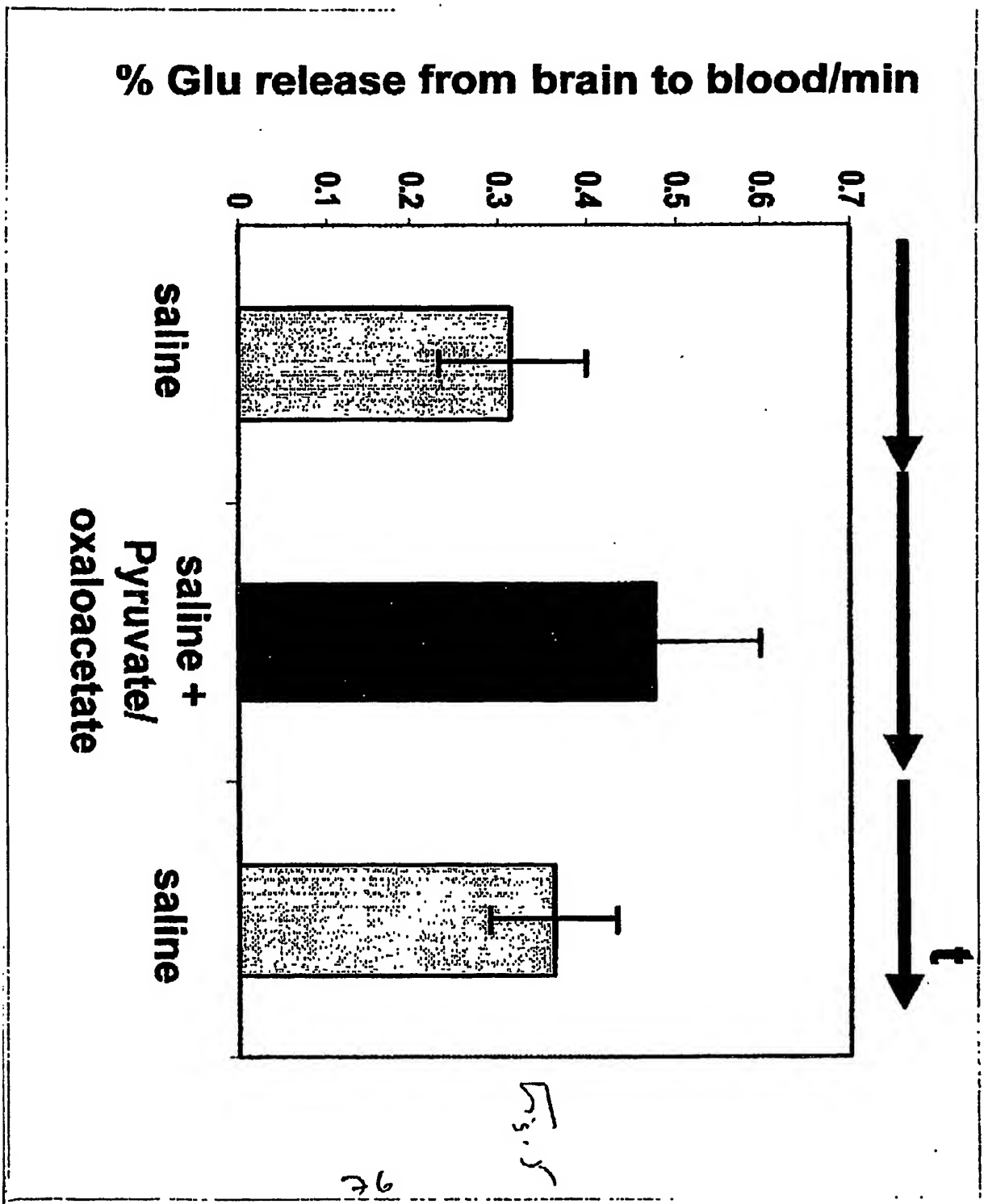
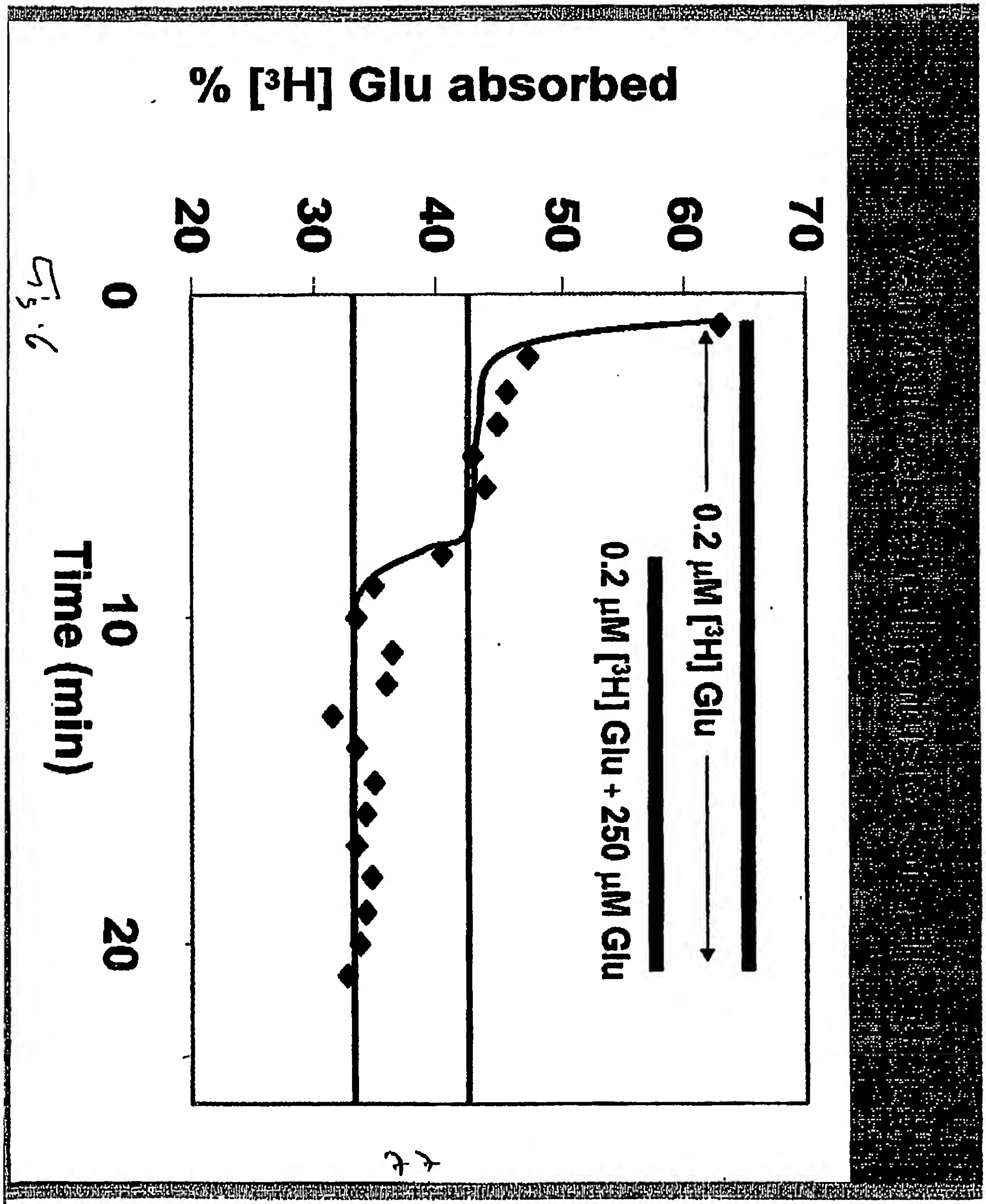

% Glu release from brain to blood/min
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
saline
saline + Pyruvate/ oxaloacetate
saline
t

FIG. 5

76

[redacted]


% $[^3H]$ Glu absorbed
20
30
40
50
60
70
0
10
20
Time (min)
0.2 µM $[^3H]$ Glu
0.2 µM $[^3H]$ Glu + 250 µM Glu

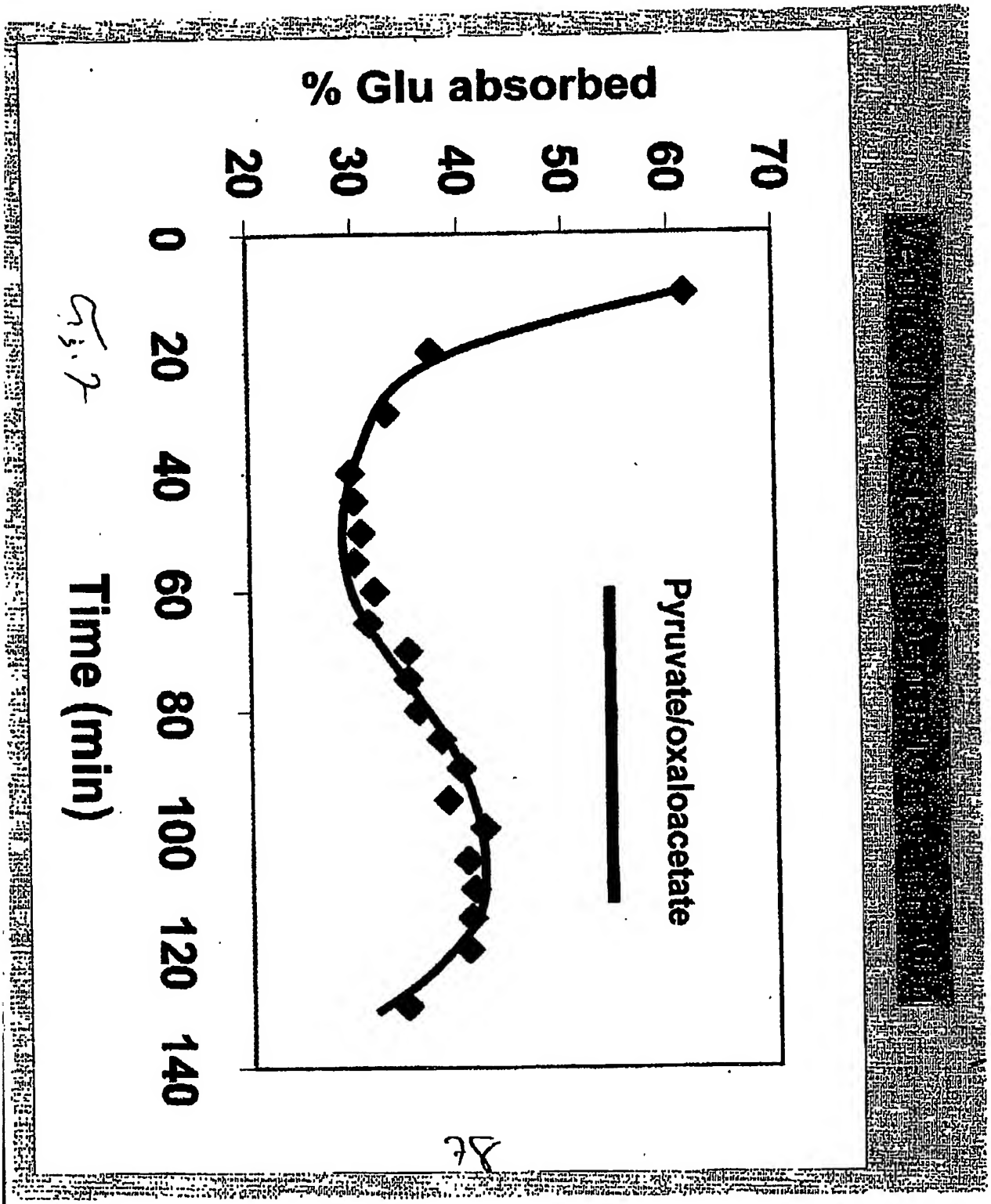

% Glu absorbed
20
30
40
50
60
70
0
20
40
60
80
100
120
140
Time (min)
Pyruvate/oxaloacetate

Fig. 2

78

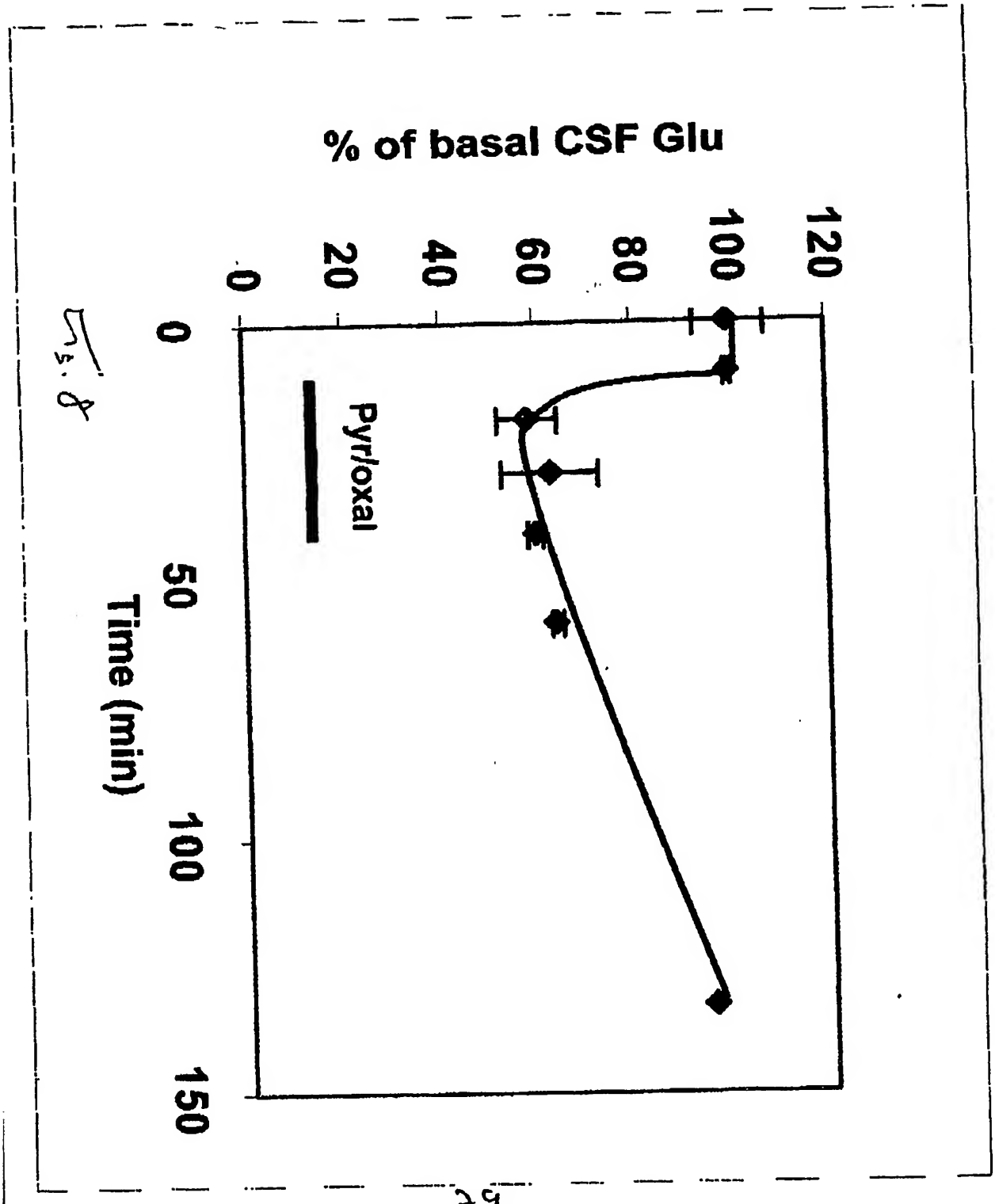

% of basal CSF Glu
0
20
40
60
80
100
120
Time (min)
0
50
100
150
Pyr/oxal

Fig. 8

Fig. 9